Cell-to-Cell Signalling in *Burkholderia cenocepacia* H111: An Entangled Web of Chemical Languages

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Summary

Burkholderia cenocepacia is a member of the *Burkholderia cepacia* complex (Bcc), a group of 17 phenotypically diverse but closely related species. Members of the Bcc are ubiquitously distributed in nature and occupy a broad range of ecological niches such as soil, water, the rhizosphere of plants, and they can be pathogens of animals and humans. This high versatility has been exploited for various purposes, including remediation of pollutants, biological control of plant pathogens and plant growth promotion. However, the great biotechnological potential of Bcc strains is severely hampered by the fact that all Bcc species have also been isolated from clinical sources, mainly from immunocompromised patients and persons suffering from cystic fibrosis (CF) or chronic granulomatous disease. Discrimination between environmental isolates and human pathogenic species is currently not possible.

The expression of pathogenic traits in *B. cenocepacia* is at least partly controlled by quorum sensing (QS). Such cell-cell communication systems are used by many bacteria to perceive and respond to the density of their population to modulate gene expression accordingly. These bacterial communication systems rely on various structurally unrelated small signalling molecules. *B. cenocepacia* H111 employs two different QS molecules. The better characterized QS system relies on *N*-acyl-homoserine lactones (AHLs). At low population densities, the cells synthesize a basal level of AHLs via the activity of the AHL synthase CepI. With increasing cell density the signal molecule accumulates and on reaching a critical threshold concentration, the signal molecule binds to its cognate receptor, CepR, which in turn activates or represses the expression of target genes.

In addition to the AHL based QS system, *B. cenocepacia* was shown to use a fatty acid derivative, *cis*-2-dodecenoic acid, as signalling molecule for the second QS system. BDSF (*Burkholderia* diffusible signal factor), is synthesized by the enoyl-CoA-hydratase $RpfF_{Bc}$. The focus of this thesis was to examine signal perception and transduction of BDSF. My work showed that *rpfR*, the gene adjacent to $rpfF_{Bc}$, encodes the main BDSF receptor in strain H111. RpfR consists of a PAS, a GGDEF and an EAL domain. The latter two are involved in c-di-GMP metabolism. RpfR is a bifunctional enzyme capable of both synthesizing and degrading c-di-GMP *in vitro*. *In vivo*, binding of BDSF to the PAS domain stimulates the phosphodiesterase activity of RpfR. The precise mechanism by which a lowered intracellular c-di-GMP concentration in turn results in altered gene expression still needs to be elucidated. But it is clear that various functions important for pathogenicity, including biofilm formation, motility and the secretion of extracellular hydrolytic enzymes, is affected. Importantly, *cepI*

expression was found to be down-regulated when *rpfR* was inactivated, providing a link between the two QS systems in *B. cenocepacia* H111. My work showed that rather than being hierarchically arranged, the two QS systems act in parallel on specific as well as overlapping sets of genes.

To further elucidate the influence of altered c-di-GMP levels on the physiology of *B. cenocepacia* H111, we artificially modified c-di-GMP levels in *B. cenocepacia* H111 and assessed the changes in global transcription patterns by RNA sequencing. I found that expression of approximately 1.5% of all genes are affected by c-di-GMP. This analysis confirmed that the c-di-GMP regulon partially overlaps with the BDSF- and the AHL-dependent QS regulons and showed that many pathogenic traits are negatively regulated by c-di-GMP. Consequently, a strain with highly increased c-di-GMP level was found to be severely attenuated in two non-mammalian infection models.

Zusammenfassung

Burkholderia cenocepacia gehört zum *Burkholderia-cepacia*-Komplex (Bcc), einer Gruppe von nah verwandten Bakterienarten, die allgegenwärtig sind und die unterschiedlichsten ökologischen Nischen, wie z.B. das Erdreich, Wasser und die Rhizosphäre besiedeln, und Tiere und Menschen infizieren können. Ihre Flexibilität wurde für verschiedene Zwecke, wie z.B. zur Bioremedation, zur biologischen Bekämpfung von Pflanzenpathogenen und für die Förderung des Pflanzenwachstums genutzt. Das grosse biotechnologische Potential von Bcc-Stämmen ist jedoch massiv beeinträchtigt durch die Tatsache, dass alle Bcc-Arten auch aus klinischen Quellen isoliert wurden. Besonders betroffen sind in diesem Zusammenhang immunsupprimierte Patienten und Personen, die an zystischer Fibrose oder an chronischer Granulomatose leiden. Eine Unterscheidung von Umweltisolaten und humanpathogenen Stämmen ist zurzeit nicht möglich.

Die Ausbildung von Pathogenitätsmerkmalen in *B. cenocepacia* ist mindestens teilweise durch einen Mechanismus namens „Quorum Sensing“ (QS) kontrolliert. Diese Art der Zell-Zell-Kommunikation wird von vielen Bakterienarten verwendet und dient der Koordination der Genexpression in Abhängigkeit von der Zelldichte der Bakterienpopulation. Bakterielle Kommunikationssysteme bedienen sich verschiedener kleiner Signalmoleküle unterschiedlichster chemischer Natur. *B. cenocepacia* H111 verwendet zwei verschieden QS-Moleküle. Das besser untersuchte QS-System beruht auf *N*-Acyl-Homoserinlactonen (AHLs). Bei einer niedrigen Populationsdichte werden aufgrund einer basalen Expression der AHL-Synthase CepI geringe Mengen an AHLs synthetisiert. Wenn die Zelldichte ansteigt, akkumulieren die Signalmoleküle, um schliesslich beim Überschreiten einer Schwellenkonzentration an den Rezeptor CepR zu binden. Der CepR-AHL-Komplex aktiviert oder reprimiert in Folge die Expression von Zielgenen.

Zusätzlich zu den AHLs verwendet *B. cenocepacia* ein Fettsäure-Derivat, *cis*-2-Dodecensäure, als Signalmolekül für das zweite QS-System. Dieses Signalmolekül, welches BDSF (*Burkholderia* diffusible signal factor) genannt wird, wird durch die Enoyl-CoA-Hydratase $RpfF_{Bc}$ synthetisiert. Der Fokus dieser Doktorarbeit lag darin, die Signal-Erkennung und -Weitergabe des BDSF QS-Systems zu untersuchen. Wir konnten *rpfR*, ein Gen das direkt neben $rpfF_{Bc}$ liegt, als BDSF-Rezeptor identifizieren. RpfR weist eine PAS-, eine GGDEF- und eine EAL-Domäne auf. Die beiden letzteren Domänen sind am Auf- und Abbau des sekundären Botenstoffes c-di-GMP beteiligt. Wir konnten zeigen, dass RpfR ein bifunktionelles Enzym ist, welches c-di-GMP sowohl synthetisieren wie auch degradieren

kann. In der Zelle führt die Bindung von BDSF an die PAS-Domäne zu einer Konformationsänderung von RpfR, wodurch es zu einer Steigerung der Phophodiesterase-Aktivität kommt. Der genaue Mechanismus, wie eine reduzierte intrazelluläre c-di-GMP-Konzentration die Expression von Genen verändert, muss noch aufgeklärt werden. Es ist jedoch klar, dass eine Reihe von Genen, die für Pathogenitätsfaktoren, wie z.B. Biofilmbildung, Motilität oder die Ausscheidung von hydrolytischen Enzymen kodieren, davon betroffen sind. Unter anderem ist die Expression von *cepI* in der *rpfR*-Mutant herunterreguliert. Dies führt zu einer Verflechtung der beiden QS-Systemen, wobei meine Untersuchungen zeigten, dass die beiden QS-Systeme nicht hierarchisch organisiert sind, sondern parallel spezifische, wie auch überlappende Sets von Genen regulieren.

Um den Einfluss von c-di-GMP auf die Physiologie von *B. cenocepacia* genauer zu untersuchen, haben wir mittels rekombinanter Plasmide die intrazelluläre c-di-GMP-Konzentration im *B. cenocepacia* H111 Wildtyp modifiziert und mittels RNA-Sequenzierung Effekte auf das Transkriptom analysiert. Mit dieser Herangehensweise identifizierten wir ca. 1.5 % aller Gene als c-di-GMP reguliert. Dies bestätigt, dass das c-di-GMP-Regulon mit den AHL- und BDSF-abhängigen Regulonen überlappt und dass viele Pathogenitätsfaktoren durch c-di-GMP negativ reguliert werden. Folgerichtig ist ein *Burkholderia*-Stamm mit massiv erhöhten intrazellulären c-di-GMP-Konzentrationen stark attenuiert in zwei Invertebraten-Infektionsmodellen.

Abbreviations

°C	degree Celsius
2-DE	two-dimensional gel-electrophoresis
AHL	*N*-acyl-homoserine lactone
BDSF	*Burkholderia* diffusible signal factor, *cis*-2-dodecenoic acid
Bcc	*Burkholderia cepacia* complex
bp	base pair
C6-HSL	*N*-hexanoylhomoserine lactone
C8-HSL	*N*-octanoyl-homoserine lactone
CAS	chrome azurol S
c-di-GMP	bis(3',5')-cyclic diguanylic acid
cDNA	complementary DNA
CF	cystic fibrosis
ddH_2O	double distilled water (Milli-Q water)
DGC	diguanylate cyclase
dH2O	deionised water
DMF	dimethyl fumarate
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxinucleotide triphosphate
DSF	diffusible signal factor
EDTA	ethylendiamintetraacetate
EPS	exopolysaccharide
EtOH	ethanol
FPLC	fast protein liquid chromatography
g	gram
GFP	green fluorescent protein
GMP	guanosine monophosphate
GTP	guanosine triphosphate
h	hour
HHQ	2-heptyl-4-quinolone
IPTG	isopropyl β-D-1-thiogalactopyranoside
kb	kilobases
kDa	kilodalton
l	liter
LC-MS/MS	liquid chromatography-tandem mass spectrometry
M	molar
MeOH	methanol
mg	milligram

min	minute
ml	milliliter
mM	millimolar
ng	nanogramm
NGM	nematode growth medium
nm	nanometer
nM	nanomolar
OD	optical density
PBE	plant-beneficial-environmental
pC3	the former chromosome 3 of Bcc species
PCR	polymerase chain reaction
PDE	phosphodiesterase
pGpG	5′-phosphoguanylyl-(3′-5′)-guanosine
pmol	picomole
PQS	Pseudomonas quinolone signal
QS	quorum sensing
RFU	relative fluorescence unit
RHL	rhamnolipid
RNA	ribonucleic acid
RNA-Seq	RNA sequencing
RPKM	reads per kilobase of transcript per million mapped reads
rpm	revolutions per minute
RT	room temperature
s	seconds
SD	standard deviation
SDS	sodium dodecyl sulphate
T3SS	type III secretion system
TCA	tricarboxylic acid
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)-aminomethan
w/v	weight per volume
Xcc	*Xanthomonas campestris* pathovar *campestris*
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	microgram
µl	microliter
µM	micromolar

1. Introduction

This introduction is in part published as a book chapter (in press):
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1.1 Members of the genus *Burkholderia* speak multiple languages

1.1.1 *N*-acyl homoserine lactone (AHL)-based QS systems in *Burkholderia* sp.

AHL-based QS systems typically rely on two proteins: an AHL synthase and an AHL receptor protein. At low cell-densities the expression of the AHL synthase gene, coding for a protein of the LuxI family, occurs at a basal level such that the signal molecules slowly accumulate in the culture medium. However, upon reaching a critical threshold concentration, the AHL molecule binds to its cognate receptor, a protein of the LuxR family of transcriptional regulators. This complex in turn binds to specific sites in the promoter regions of target genes, activating or repressing their transcription (Williams, 2007). First indications that a *Burkholderia* species produces AHLs were obtained in a study that showed that the culture supernatant of a *B. cepacia* strain was able to activate different AHL biosensors (McKenney *et al.*, 1995). A genetic screen for mutants with altered siderophore production in *Burkholderia cenocepacia* K56-2, eventually lead to the identification of the CepI/R system (Lewenza *et al.*, 1999). The AHL synthase, CepI, was shown to direct the synthesis of *N*-octanoyl-homoserine lactone (C8-HSL) and minor amounts of *N*-hexanoylhomoserine lactone (C6-HSL) (Figure 1). Subsequent studies revealed that the CepR/AHL complex binds as a dimer to an imperfect palindromic sequence that often partially overlaps the -35 regions of target promoters, thereby initiating transcription of downstream genes (Chambers *et al.*, 2006; Wei *et al.*, 2011). This recognition sequence, referred to as *cep* box, is also present in the promoter region of *cepI*. Thus, once the QS system is triggered a positive feedback loop is established that leads to a rapid increase in AHL levels and consequently in target gene expression. In addition, the CepR/AHL complex also negatively controls its own expression (Lewenza & Sokol, 2001).

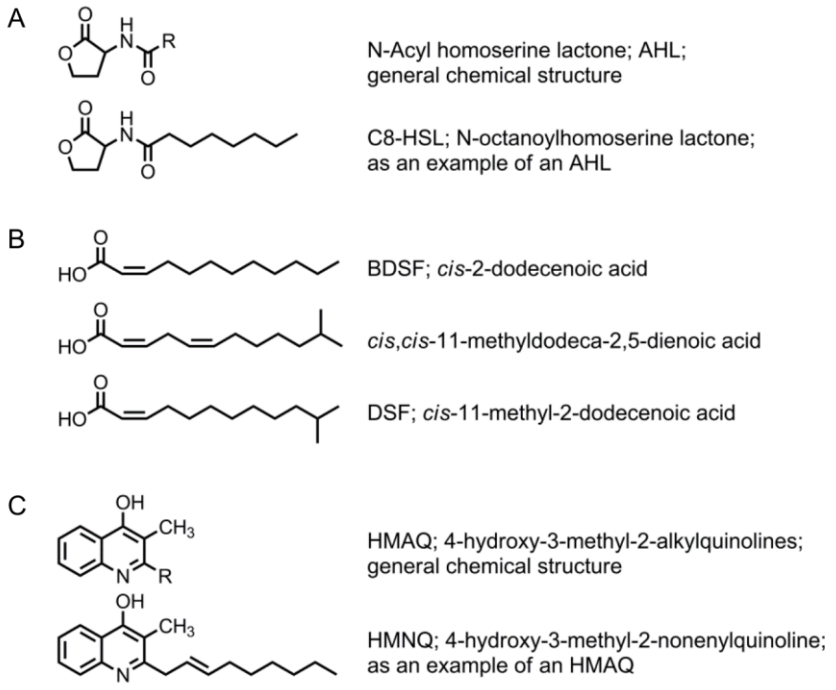

Figure 1. The different QS signalling molecules used by Burkholderia species. (A) Signalling molecules in AHL-based QS. (B) Signalling molecules in fatty acid based QS. (C) Signalling molecules in quinoline-based QS.

The CepI/R system appears to be present in all 17 *Burkholderia cepacia* complex (Bcc) species, although direct experimental evidence is missing for a few of them, namely *B. arboris, B. diffusa, B. latens, B. metallica,* and *B. seminalis* (Gotschlich *et al.*, 2001; Venturi *et al.*, 2004; Wopperer *et al.*, 2006). CepI/R homologs have also been identified in all members of the pseudomallei group, consisting of *B. pseudomallei*, *B. mallei*, *B. thailandensis* and *B. oklahomensis,* as well as in in some species of the recently defined plant-beneficial-environmental *Burkholderia* group (PBE), although it remains to be seen whether they belong to independent evolutionary lineages (Suárez-Moreno *et al.*, 2010). Recent work has also provided evidence that many members of the PBE group harbour another highly conserved LuxIR pair, designated BraI/R, that relies on the AHL signal 3-oxo-C14-HSL (Suárez-Moreno *et al.*, 2008). Finally, many *Burkholderia* strains have been demonstrated to harbour more than one AHL-dependent QS system and produce multiple AHL signal molecules (Riedel & Eberl, 2007). It is beyond the scope of this chapter to summarize all these systems and I will rather focus on *B. cenocepacia*, which is the model organism of this work..

In addition to the CepI/R system, *B. cenocepacia* strains belonging to the ET12 lineage carry the CciI/R QS system on the *B. cenocepacia* island (cci), a 31.7-kb low GC content pathogenicity island encoding 35 putative ORFs (Baldwin *et al.*, 2004). This QS system

differs from the CepI/R system in that *cciI* and *cciR* form a transcriptional unit, whereas *cepI* and *cepR* are divergently transcribed (Malott *et al.*, 2005). Furthermore, the main product of CciI is C6-HSL, while C8-HSL, the main product of CepI, is only produced in minor amounts (Malott *et al.*, 2005). The CepI/R QS system and the CciI/R QS system do not act independently: CepR is required for transcription of the *cciIR* operon, whereas CciR negatively controls the expression of CepI, but not of CepR (Malott *et al.*, 2005). Results from a transcriptomic analysis suggested a reciprocal regulation of target genes by the two QS systems, which ensures fine-tuned expression of target genes (O'Grady *et al.*, 2009).

Over the past few years it has become evident that the *B. cenocepacia* QS circuitry is far more complex than initially anticipated, as various higher-level regulatory elements as well as downstream regulators were identified. An additional regulatory element influencing the activity of the CepI/R QS system is ShvR, a LysR type transcriptional regulator that was initially shown to be responsible for shiny colony variants of *B. cenocepacia* K56-2 (Bernier *et al.*, 2008). In a recent study it has been shown that ShvR controls the expression of more than 1000 genes (O'Grady *et al.*, 2011), including *cciI*/*R* and *cepI*/*R,* although the effect on the latter system was only weak. Consequently, ShvR affects AHL kinetics: maximal AHL production peaked earlier in a *shvR* mutant compared to wild type (O'Grady *et al.*, 2011). However, the regulons of ShvR and CepR/CciR only partially overlap and phenotypic analyses of mutants defective in *shvR*, *cepR* or *cciR* suggest that ShvR not only influences expression of CepR- and CciR-regulated genes, but also of a set of genes which is not under direct QS-control (O'Grady *et al.*, 2011).

In addition to ShvR, another global regulator, AtsR, was shown to modulate QS signalling. This response regulator was first identified as a repressor of biofilm formation and was shown to control expression of a type VI secretion system (Aubert *et al.*, 2008). A recent study demonstrated that deletion of *atsR* led to increased expression of *cepI/R* and *cciI/R*, which in turn led to premature and increased AHL production. This finding suggests that AtsR plays a role in controlling the timing and fine-tuning of AHL-dependent gene expression. However, AtsR is also able to repress QS-regulated genes independently of AHL production, indicating that AtsR may operate *via* a novel yet uncharacterized mechanism (Aubert *et al.*, 2012).

In contrast to ShvR and AtsR, which down-regulate both QS systems, a recent study identified a regulator that promotes transcription of *cepI/R* and *cciI/R* in *B. cenocepacia* K56-2 (O'Grady *et al.*, 2012). This regulator, BCAM1871, is co-transcribed with *cepI* and acts as an enhancer of AHL production (positive feedback). Mutants in BCAM1871 showed reduced

transcription levels of *cciIR* and of *shvR*. However, and similar to AtsR, some phenotypes were affected independently of AHL levels or CepI/R function, suggesting that BCAM1871 may operate through an AHL-independent mechanism (O'Grady *et al.*, 2012).

As many other Proteobacteria, *B. cenocepacia* harbors an orphan or solo *luxR* gene, i.e. a *luxR* gene not associated with a cognate *luxI* AHL synthase gene (for a review see (Subramoni & Venturi, 2009).This LuxR solo, designated CepR2, is a 237 amino-acid ortholog of CepR and contains all the conserved residues of LuxR transcriptional regulators (Malott *et al.*, 2009). In *B. cenocepacia* K56-2, CepR2 is negatively regulated by CciR and by itself, and it does not affect expression of the CepI/R or the CciI/R system. CepR2 was shown to be both a negative and a positive regulator of gene expression and does not require AHLs to exert its function (Malott *et al.*, 2009). Interestingly, the set of genes regulated by CepR2 in strain K56-2 partially overlaps with those of the CepI/R and CciI/R regulons, including the genes encoding the metalloproteases ZmpA and ZmpB and the nematocidal factor AidA.

1.1.2 Fatty acid signalling in *Burkholderia* sp.

In 2008, Boon *et al.* discovered that some *Burkholderia* strains produce a long chain fatty acid signal molecule, the structure of which was identified as *cis*-2-dodecenoic acid, referred to as BDSF (*Burkholderia* diffusible signal factor). BDSF is a structural and functional analogue of *cis*-11-methyl-2-dodecenoic, the diffusible signal factor (DSF) of *Xanthomonas campestris* pv. *campestris* (Xcc), which is a well characterized QS signal in this organism. Analyses of structural derivatives of DSF revealed that the key features of this type of QS signal molecules in Xcc are the double bond between C1 and C2, as well as its *cis*conformation (Wang *et al.*, 2003), both features are present in BDSF. Genetic analysis revealed that in *B. cenocepacia* J2315, the gene responsible for BDSF biosynthesis is BCAM0581. This gene codes for an enoyl-CoA hydratase that shares 37% identity at the amino acid level with RpfF, the main DSF synthase of Xcc. *In trans* expression of BCAM0581 in a DSF-deficient Xcc strain can rescue the defects of the mutant, including biofilm formation and extracellular polysaccharide production (Boon *et al.*, 2008). BCAM0581 has been recently renamed to $rpfF_{Bc}$ and shown to encode a bifunctional enzyme that catalyses the dehydration of 3-hydroxydodecanoyl-ACP to *cis*-2-dodecenoyl-ACP and cleaves the thioester bond to give BDSF (Bi *et al.*, 2012). Besides BDSF, some *Burkholderia* strains also produce additional DSF-family signals like DSF or *cis,cis*-11-methyldodeca-2,5-dienoic acid (DSF with an additional double bond in *cis*-configuration between C5 and C6) (Deng *et al.*, 2010).

Examination of nine species from the Bcc (*B. lata*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina* and *B. pyrrocinia*) showed that all produced BDSF, with four of them (*B. multivorans*, *B. stabilis*, *B. anthina*, and *B. pyrrocinia*) additionally producing *cis,cis*-11-methyldodeca-2,5-dienoic acid and *B. multivorans* producing DSF (Deng *et al.*, 2010). Interestingly, the observed diversity of DSF-family signal molecules in Bcc species is not related to variations found in the $RpfF_{Bc}$ primary structure, but is likely due to the availability of different precursors in the different Bcc species (Deng *et al.*, 2010). Blast analyses revealed orthologues of $RpfF_{Bc}$ from *B. cenocepacia* J2315 in all members of the *pseudomallei* group, in two phytopathogenic *Burkholderia* species (*B. glumae* and *B. rhizoxinica*) and in members of the PBE cluster (*B. graminis*, *B. phymatum*, *B. phytofirmans*, *B. terrae* and *B. xenovorans*) (Deng *et al.*, 2012; Winsor *et al.*, 2008).

Although Xcc is using a similar signal molecule as *B. cenocepacia*, the signal receptor as well as the signalling cascade is completely different. The DSF receptor of Xcc, RpfC, is a hybrid sensor kinase that phosphorylates its cognate response regulator RpfG. In addition to a receiver domain RpfG contains a HD-GYP domain, which was shown to be responsible for the c-di-GMP phosphodiesterase activity of the protein thereby affecting the cellular c-di-GMP level (Ryan & Dow, 2010). However, neither *rpfC* nor *rpfG* orthologs could be identified in any of the sequenced Bcc genomes.

1.1.3 Quinolone-based QS in *Burkholderia* sp.

In addition to AHL- and BDSF-based QS systems, a third potential QS system, which relies on quinolone signal molecules, was identified in some *Burkholderia* strains. The archetypal quinolone-based QS system is the one of *Pseudomonas aeruginosa,* which utilizes 2-heptyl-3-hydroxy-4(1*H*)-quinolone (also known as the Pseudomonas quinolone signal, PQS) to control expression of many virulence genes. PQS is also considered an integral component of the AHL-dependent QS network of this organism (Diggle *et al.*, 2006). The biosynthesis of PQS relies on the *pqsABCD* gene products, which direct the synthesis of 2-heptyl-4-quinolone (HHQ), the precursor of PQS synthesis. HHQ is thought to be an extracellular messenger that is released from, and taken up by, bacterial cells, where it is converted into PQS. Using LC-MS/MS analysis it was shown that several *B. pseudomallei* strains, *B. cenocepacia, B. thailandensis* and *B. ambifaria* produce, in addition to HHQ, other 4-hydroxy-2-alkylquinolone derivatives and also 4-hydroxy-3-methyl-2-alkylquinolones but not PQS (Diggle *et al.*, 2006; Vial *et al.*, 2008). Mutants of *B. ambifaria* defective in quinolone

signalling were shown to have an increased production of AHLs and, consequently, slightly up-regulated expression of AHL-dependent phenotypes (Vial *et al.*, 2008). In *B. pseudomallei* mutation of the *pqsA* homologue not only resulted in the loss of HHQ production but also in altered colony morphology and increased elastase production (Diggle *et al.*, 2006). Additional work will be required to characterize the quinolone-dependent signalling cascade and how it may interact with the AHL- and BDSF-dependent QS circuitries.

1.2 QS-regulated phenotypes and regulons

The impact of QS on the physiology of *Burkholderia* species has been assessed by phenotypic characterization as well as by global profiling approaches (transcriptomes or proteomes) using defined QS mutants. The majority of these studies focused on pathogenic species, including Bcc species, the pseudomallei group and the rice pathogen *B. glumae*. In Table 1 we show QS-regulated phenotypes of different *Burkholderia* species. Some of the listed functions, including biofilm formation, proteolytic activity, motility, antifungal activity and pathogenicity are highly conserved, suggesting that they represent ancestral QS-regulated phenotypic traits.

Table 1. Experimentally determined phenotypes of QS mutants in Burkholderia species.

Species[a]	Mutated QS system	Biofilm[c]	Protease	Chitinase Lipase	Siderophore	Swarming motility	Swimming motility	Antifungal activity	Pathogenicity: *C. elegans*	Pathogenicity: *Galleria*	Pathogenicity: mouse/rat/hamster	Pathogenicity: zebrafish	Pathogenicity: alfalfa	Other QS regulated phenotypes	Reference:
B. ambifaria	*cepI/R*	↓[d]	↓					↓	↓	↓					(Uehlinger *et al.*, 2009; Wopperer *et al.*, 2006)
	HMAQ		↑		↑			↑							(Vial *et al.*, 2008)
B. anthina	*cepI/R*	↓	↓ (1), – (1)			↓			↓						(Wopperer *et al.*, 2006)
B. cenocepacia with *cciIR*	*cepI/R*	↓	↓	↓	↑	↓	↓		↓	↔	↓	↓	↔		(Lewenza *et al.*, 1999; Malott *et al.*, 2005; O'Grady *et al.*, 2009; Sokol, 2003; Tomlin *et al.*, 2005; Uehlinger *et al.*, 2009; Vergunst *et al.*, 2010)
	cciI/R	↓ (cciR), ↔ (cciI)	↑ (cciI), ↓ (cciR)		↔	↓ (cciI), ↔ (cciR)	↑		↔	↔	↓		↔		(Malott *et al.*, 2005; O'Grady *et al.*, 2009; Tomlin *et al.*, 2005; Uehlinger *et al.*, 2009)
	rpfF/R	↓				↓				↓	↓	↓		Germtube formation in *Candida albicans*:↓ ; adherence to porcine mucin: ↓, cytotoxicity: ↓	(Boon *et al.*, 2008; Deng *et al.*, 2009; McCarthy *et al.*, 2010; Ryan *et al.*, 2009)
B. cenocepacia without *cciIR*	*cepI/R*	↓	↓	↓ ↔	↑	↓	↔	↓	↓	↓ cepI , ↔ cepR	↓, ↔[f]				(Huber *et al.*, 2001; Köthe *et al.*, 2003; Schmidt *et al.*, 2009; Sousa *et al.*, 2007; Uehlinger *et al.*, 2009)
	rpfF/R	↓	↓			↓			↓						(Deng *et al.*, 2012)
B. cepacia	*cepI/R*	↓	↓	↔↔	↔			↓	↓					Onion pathogenicity: ↓	(Aguilar *et al.*, 2003; Schmidt *et al.*, 2009; Wopperer *et al.*, 2006)
B. dolosa	*cepI/R*	↓	–						↓ (1), – (1)						(Wopperer *et al.*, 2006)
B. lata	*cepI/R*				↔			↓							(Schmidt *et al.*, 2009)
B. multivorans	*cepI/R*	↓	–						– (6), ↓(1)						(Wopperer *et al.*, 2006)
B. pyrrocinia	*cepI/R*	↓(1), ↑ (1)	↓					↓	↓						(Schmidt *et al.*, 2009; Wopperer *et al.*, 2006)
B. stabilis	*cepI/R*	↓	↓			↓		↓	↓						(Schmidt *et al.*, 2009; Wopperer *et al.*, 2006)
B. vietnamiensis	*cepI/R*	↓(3), ↑(2), ↔ (1)	–	–	↔	–		↓	↓ (3), – (2)	↓			–		(Conway & Greenberg, 2002; Schmidt *et al.*, 2009; Uehlinger *et al.*, 2009; Wopperer *et al.*, 2006)
	bviI/R	↔	–	–	↔	–							–		(Malott & Sokol, 2007)

B. pseudomallei	*pmlI/R* *bpsI/R*	or ↓	↑, ↔ [e]	↔	↑			↓	↓	Sensitivity to oxidative stress: ↑	(Gamage *et al.*, 2011; Lumjiaktase *et al.*, 2006; Song *et al.*, 2005; Ulrich *et al.*, 2004a; Valade *et al.*, 2004)
B. thailandensis	*btaI/R*		↔	↓, ↑	↔	↑			↔	Bactobilin synthesis ↓, selfaggregation ↓	(Chandler *et al.*, 2009; Duerkop *et al.*, 2009; Seyedsayamdost *et al.*, 2010; Ulrich *et al.*, 2004b)
B. mallei	*bmaII/R*		↔	↔					↓		(Ulrich *et al.*, 2004c)
B. glumae	*TofI/R*			↓		↓	↓			Catalase: ↓, toxoflavin synthesis: ↓, heat shock resistance ↓, rice pathogenicity: ↓	(Chun *et al.*, 2009; Devescovi *et al.*, 2007; Kim *et al.*, 2012, 2004, 2007)
B. plantarii	*PlaI/R*									rice seedling blight ↓	(Solis *et al.*, 2006)
B. kururiensis	*BraI/R*	↑	↔	↔	↔	↔	↔			EPS production: ↓	(Suárez-Moreno *et al.*, 2008, 2010)
B. xenovorans	*BraI/R*	↔	↔	↔	↔	↔	↔			EPS production: ↓	(Suárez-Moreno *et al.*, 2008, 2010)
B. unamae	*BraI/R*	↑								EPS production: ↓, phenol degradation: ↓	(Suárez-Moreno *et al.*, 2008, 2010)
B. caryophylii	AHL[b]						↓				(Schmidt *et al.*, 2009)
B. phenazinium	AHL[b]						↓				(Schmidt *et al.*, 2009)
B. bryophila	AHL[b]						↓				(Schmidt *et al.*, 2009)
B. megapolitana	AHL[b]						↓				(Schmidt *et al.*, 2009)

[a] *Burkholderia* species, in which a QS regulated phenotype has been observed.
[b] The phenotypic testing has been performed with an AHL degrading approach.
[c] Number in parentheses indicates the number of strains in which the according regulation has been observed if a phenotype is strain-dependent.
[d] ↓ down-regulated in QS mutant, ↑ up-regulated in QS mutant, ↔ not regulated in QS mutant (experimentally verified), – phenotype not observed in wild-type strain.
[e] Opposing observations in different studies.
[f] Reciprocally by the different QS systems: btaI/R1 and btaI/R3 ↑, btaI/R2.

QS-regulated functions were also mapped by functional genomics approaches. A transcriptomic analysis of CepR-regulated genes in *B. cenocepacia* H111 identified 57 genes that were more than threefold up- or down-regulated in a *cepR* mutant relative to the wild-type strain (Inhülsen *et al.*, 2012). The same custom *B. cenocepacia* oligonucleotide microarray was also used to identify the overlap between the CepI/R and CciI/R systems in *B. cenocepacia* K56-2 (O'Grady *et al.*, 2009). When compared with the wild type, 860 genes were found to be more than twofold up- or down-regulated in a K56-2 *cepR* mutant, 595 genes in a *cciR* mutant and 489 genes in a *cepR cciR* double mutant (O'Grady *et al.*, 2009). Among those genes, 196 genes were regulated by both CepR and CciR, with the vast majority (167) being positively regulated by CepR and negatively regulated by CciR. With only very few exceptions all 57 CepR-regulated genes identified in strain H111 (Inhülsen *et al.*, 2012) were also among the CepR-regulated genes of strain K56-2. Both studies showed that *aidA*, which encodes a factor required for nematode pathogenicity, was among the most stringently CepR-activated genes. Interestingly CciR had a negative effect on *aidA* transcription in strain K56-2. The dual regulation of *aidA* in K56-2 provides an explanation for the observation that the *cepI* mutants of H111 and K56-2 but not the K56-2 *cciI* mutant are attenuated in virulence in the *C. elegans* infection model (Uehlinger *et al.*, 2009). Other AHL-regulated genes that were identified in both studies include several proteases, an operon encoding three lectins, a type I pilus, a non-ribosomal peptide synthetase gene cluster, and genes required for swimming motility (Inhülsen *et al.*, 2012; O'Grady *et al.*, 2009).

Global proteomic analyses have been performed to study the QS regulon of *B. cenocepacia* H111. An early study by Riedel *et al.* (2003) employed two-dimensional gel-electrophoresis (2-DE) to compare the proteomes of the H111 wild type and its isogenic *cepI* mutant grown in presence or absence of AHLs. Fifty five of the 985 detected protein spots were found to be differentially regulated. Due to limited genome sequence data at that time, only 19 of the 55 QS-regulated proteins could be identified. In good agreement with more recent transcriptomic data (Inhülsen *et al.*, 2012; O'Grady *et al.*, 2009), the proteins included the ZmpB metalloprotease, the FimA protein, the BclA lectin and AidA. A more recent study, employing a gel-free proteomics methodology, identified 22 proteins that were down-regulated in a *cepI* as well as in a *cepR* mutant of *B. cenocepacia* H111 (Inhülsen *et al.*, 2012). This analysis not only provided confirmatory results but also showed that many of the QS-regulated proteins were not among the QS-regulated genes identified by the microarray analyses, suggesting that

expression of many of the identified proteins may be controlled by QS at the posttranscriptional level.

2-DE was also employed to identify AHL-regulated proteins in *B. glumae* BGR1 (Goo *et al.*, 2010). Among 79 differentially expressed proteins identified, 59 were extracellular proteins, such as flagellin, lipases, proteases, proteins involved in anti-oxidation and membrane proteins. The high proportion of extracellular proteins among the QS-regulated proteins was elegantly explained by the finding that expression of the type II secretion system, which is required for secretion of many extracellular proteins, is controlled by QS (Goo *et al.*, 2010). Likewise, 2-DE was used to map the proteins controlled by one (the *bpsI/R* system) of the three AHL-dependent QS systems in *B. pseudomallei* (Wongtrakoongate *et al.*, 2012). Forty five of the 65 identified genes were found to be also regulated by the stationary sigma factor RpoS, most likely because RpoS is involved in the regulation of *bpsI* expression.

1.3 Molecular mechanisms and factors important for biofilm formation of *Burkholderia* sp.

One of the phenotypes regulated by both the AHL- and BDSF-dependent QS system (no data on the role of quinolone signalling are available) is the formation of surface-associated communities, referred to as biofilms. In nature bacteria are thought to exist predominantly as biofilms on both biotic and abiotic surfaces. Biofilms are also of eminent clinical importance, as sessile cells are up to 1000-fold more resistant to antibiotics than their planktonic counterparts. In the following we will discuss factors that have been reported to play a role in biofilm formation of *Burkholderia* sp.

1.3.1 Pili

The adhesion to surfaces is often mediated by specialized proteinaceous surface appendages such as fimbriae or pili or in some bacteria also flagella (Watnick & Kolter, 2000). *Burkholderia* sp. expresses flagella and various types of pili (Goldstein *et al.*, 1995). Using transmission electron microscopy (TEM), five types of pili were identified in Bcc strains: mesh (Msh), filamentous (Fil), spine (Spn), spike (Spk), and cable (Cbl) pili. The expression of these different pili types has been linked to the ecological niches from which the respective strains have been isolated from. Msh pili have been shown to be produced by many clinical and environmental strains, whereas Cbl pili were only expressed by epidemic CF isolates. Fil

pili were found to be associated with non-epidemic CF isolates, Spn pili with non-CF clinical isolates, and Spk pili were exclusively produced by environmental strains. The cable pilus Cbl has been demonstrated to be important for adhesion of Bcc strains to epithelial cells and is therefore thought to be of particular clinical relevance (Sajjan *et al.*, 2002). It is noteworthy that only a few Bcc strains harbour the gene coding for the Cbl pilus and not all strains possessing the gene produce pili (Sajjan *et al.*, 2002).

Many *Burkholderia* strains produce a homologue of the *E. coli* FimA protein, the major subunit of the type I pilus. In *E. coli* this pilus is critical for initial attachment to biotic and abiotic surfaces (Pratt & Kolter, 1998). A FimA homolog in *B. cenocepacia* H111 was identified among the CepR-regulated genes (Riedel *et al.*, 2003). *fimA* (BCAL1677) is the first gene of an operon that also contains genes encoding for a chaperone-usher secretion apparatus (BCAL1678-1681) (Holden *et al.*, 2009; Inhülsen *et al.*, 2012). Regulatory elements like homologs of FimB and FimE, which control phase variation in *E. coli*, or a tip adhesin are missing in *B. cenocepacia* H111. In spite of the fact that type I fimbriae are crucially important in some organisms for adhesion to surfaces, a *fimA* mutant of *B. cenocepacia* H111 was neither defective in biofilm biomass nor in biofilm architecture (Inhülsen *et al.*, 2012). As the analysis of the H111 genome indicated the presence of at least two additional chaperon-usher-type pili, two Flp-type pili, a type IV pilus, it is conceivable that these additional surface appendages may mask the biofilm defects of the *fimA* mutant. In *B. pseudomallei*, the type IV pilin-encoding gene *pilA* was shown to be essential for microcolony development, and thus optimum association with eukaryotic cells, but is not required for direct adherence to cultured human cells (Boddey *et al.*, 2006).

1.3.2 Genes required for biofilm maturation

Using a simple microtiter dish based assay Huber *et al.* (2002) screened a random transposon insertion library of *B. cenocepacia* H111. Given the relatively long incubation time (48 hours), mutants defective in the late steps of biofilm formation were enriched in this screen. Of 5000 transposon insertion mutants screened, 13 exhibited defects in biofilm formation on a polystyrene surface without being impaired in growth. However, all the mutants were defective in the development of the typical three-dimensional biofilm structure of *B. cenocepacia* H111. The 13 mutants were shown to carry the transposon in genes encoding

surface proteins, proteins involved in the biogenesis and maintenance of an integral outer membrane, regulators or proteins involved in QS (Huber *et al.*, 2002).

1.3.2.1 Bacterial surface proteins

In two of the transposon mutants a gene encoding a large surface protein was inactivated. This gene was named *bapA* (biofilm associated protein A). Various members of this protein family were shown to be involved in the colonization of diverse substrates: Mus-20 (mutants unattached to seeds) in the adhesion of *P. putida* KT2440 to corn seeds (Espinosa-Urgel *et al.*, 2000), Bap of *Staphylococcus aureus* in biofilm formation on mammary gland epithelium of ruminants (Cucarella *et al.*, 2001), Esp (Enterococcal surface protein) of *Enterococcus faecalis* in human skin colonization (Toledo-Arana *et al.*, 2001), *lapA* (large adhesion protein) of *P. fluorescens* in biofilm formation on abiotic surfaces (Hinsa *et al.*, 2003), and BapA of *Salmonella enterica* in air-broth interface pellicle and biofilm formation (Latasa *et al.*, 2005). These large proteins are usually secreted via a type I secretion system and are believed to remain loosely associated with the cell surface (Hinsa *et al.*, 2003; Latasa *et al.*, 2005). In a more recent study, a *bapA* mutant was found to be severely impaired in biofilm formation on an abiotic surface and showed defects in terms of attached biofilm mass as well as biofilm architecture (Inhülsen *et al.*, 2012). BapA was shown to be exported via a type I secretion system. The three genes encoding this export machinery are located downstream of *bapA* and are co-transcribed with *bapA* as a single transcription unit. A mutant with a mutated type I transporter exhibited the same biofilm defect as a *bapA* mutant and microscopic inspection of a strain expressing a BapA-mCherry fusion protein suggested that BapA is no longer exported (Inhülsen *et al.*, 2012).

Although not identified in the mutant screen, an operon encoding three lectins (*bclACB*, BCAM0186 to BCAM0184) was found to influence biofilm structural development (Inhülsen *et al.*, 2012). All three lectins share an almost identical PA-IIL-like C-terminal domain; BclB and BclC have additional N-terminal domains. PA-IIL is a soluble lectin from *P. aeruginosa* with a strong affinity to fucose. A *P. aeruginosa* mutant not expressing PA-IIL is impaired in biofilm formation on glass slides when compared to the wild-type strain (Tielker *et al.*, 2005; Winzer *et al.*, 2000). Two of the three lectins of *B. cenocepacia*, BclA (BCM0186) and BclC (BCAM0185), were the subject of recent studies (Lameignere *et al.*, 2008, 2010; Marchetti *et al.*, 2012; Sulák *et al.*, 2010, 2011). These investigations revealed that BclA forms

homodimers and displays a strict specificity for oligomannose-type oligosaccharides that are present on human glycoproteins (Lameignere *et al.*, 2008; Marchetti *et al.*, 2012). Moreover, it was shown that BclA binds to bacterial surfaces and to biofilms (Marchetti *et al.*, 2012). BclC, which also contains the C-terminal PA-IIL domain, possesses in addition an N-terminal domain comprising a TNF-α-like fold with fucose binding properties. BclC forms hexamers and, due to its two different lectin domains, is a super lectin with dual carbohydrate specificity (Sulák *et al.*, 2010, 2011). BclB is the least characterized of the three lectins encoded by *B. cenocepacia*. In a recent study it was shown that BclB is associated with the bacterial cell surface (Inhülsen *et al.*, 2012). This study also revealed that the structure of the biofilm formed by a *bclACB* mutant is different from the one of the wild type. The mutant biofilm exhibited a very characteristic biofilm architecture structure with hollow microcolonies, which could not be observed with the wild type. This change in biofilm morphology could only be rescued when the complementation was done with the intact *bclACB* operon, suggesting that the three lectins are not redundant and that all three lectins are needed for biofilm structural development. It has been suggested that the surface-exposed BclACB lectins may mediate contact to neighboring cells within the biofilm or with the biofilm matrix.

1.3.2.2 Proteins involved in the biogenesis and maintenance of an integral outer membrane

Two genes essential for maintaining cell shape and outer membrane composition, *tolA* and *rodA,* were found to be required for biofilm formation. In *P. aeruginosa*, the homologue of *tolA* affects LPS structure and its expression was shown to be induced during biofilm formation (Whiteley *et al.*, 2001). In addition, *tolA* is involved in the biogenesis and maintenance of an integral outer membrane in both *E. coli* and *P. putida* (Lazzaroni *et al.*, 1999; Llamas *et al.*, 2000). In agreement with this findings, *tolA* mutants of *B. cenocepacia* H111 were elongated and grew in chains, forming flat and unstructured biofilms (Huber *et al.*, 2002). In contrast to *tolA* mutants, *rodA* mutants are coccoid and form hyper-structured biofilms with big microcolonies. Taken together, these data suggests that cell shape and cell surface composition are important factors for biofilm development.

1.3.2.3 Exopolysaccharide production

Exopolysaccharides (EPS) are considered to be an essential constituent of the biofilm matrix, even though the genes required for EPS biosynthesis were not identified in the mutant screen of Huber *et al.* (2002). The majority of Bcc strains produce large amounts of EPS and so far five different EPS molecules have been identified (Herasimenka *et al.*, 2007). Among them, cepacian, a polysaccharide with a branched heptasaccharide repeating unit, is the most abundant EPS produced by Bcc species (Herasimenka *et al.*, 2007). It has been shown that cepacian plays a role in biofilm maturation, but is not required for the initiation of biofilm formation (Cunha *et al.*, 2004). The enzymes required for cepacian biosynthesis are encoded by two separated gene clusters, *bce-I* and *bce-II* (Moreira *et al.*, 2003; Ferreira *et al.*, 2010). Interestingly, due to a frameshift mutation in the *bceB* gene (in the *bce-I* cluster), the ET12 lineage strain *B. cenocepacia* J2315, which is used as model strain in many laboratories, does not produce cepacian (Holden *et al.*, 2009). Despite the fact that bacteria growing in biofilms have been shown to be more resistant to both antibiotics and phagocyte killing than their planktonic counterparts (Høiby *et al.*, 2010), no clear correlation between cepacian production and the clinical outcome of the infection could be established when CF isolates of *B. cepacia*, *B. multivorans*, *B. cenocepacia*, and *B. stabilis* were analysed (Cunha *et al.*, 2004).

Zlosnik *et al.* (2008) reported an inverse correlation between the quantity of mucoid exopolysaccharide production by Bcc bacteria and rate of decline in CF lung function, which is in contrast to what is known from *P. aeruginosa*. It has been suggested that non-mucoid isolates are associated with increased disease severity while the mucoid phenotype may be associated with bacterial persistence. In agreement with this hypothesis, it was shown that EPS from a clinical *B. cenocepacia* isolate inhibited the chemotaxis of neutrophils and scavenges reactive oxygen species, both essential components of innate neutrophil-mediated host defences (Bylund *et al.*, 2006). In addition, it is possible that the loss of EPS production provides the bacteria with a competitive advantage in the lung and/or allows for increased virulence factor production (Zlosnik *et al.*, 2008).

1.3.3 The role of QS in biofilm formation

A role for AHL-mediated QS in regulation of biofilm formation was first reported by Davies *et al.* (1998) for *P. aeruginosa*. Specifically, it was shown that a QS mutant formed flat and

undifferentiated biofilms when compared to the wild-type biofilm. Various QS-regulated functions, including the biosynthesis of rhamnolipids, the production of the biofilm matrix polysaccharide Pel, anaerobic denitrification, and *P. aeruginosa* quinolone signal (PQS)-dependent DNA release, have been identified as obvious links between biofilm structural development and cell–to-cell communication (for reviews, see Aguilar *et al.*, 2009; de Kievit, 2009). Recent studies have shown that QS is necessary for the formation of the cap portion of the mushroom-shaped microcolonies, which are typical of *P. aeruginosa* biofilms (Patriquin *et al.*, 2008; Yang *et al.*, 2007, 2009). However, it is noteworthy that in studies using slightly changed experimental settings or hydrodynamic conditions, no significant differences between biofilms of the wild type and those formed by QS-negative mutants were observed (Heydorn *et al.*, 2002; Schaber *et al.*, 2007; Stoodley *et al.*, 1999), suggesting that different experimental settings have a strong influence on expression of QS-regulated genes.

B. cenocepacia H111 was the second example of a bacterium that requires a functional AHL-dependent QS system for the formation of differentiated biofilms (Huber *et al.*, 2001). While both wild type and mutants defective in the CepI/R QS system form characteristic microcolonies after initial attachment to an abiotic surface, striking differences can be observed in the later stages of biofilm development: the wild-type biofilm rapidly matures whereas the QS-defective strains are arrested at the microcolony stage and thus form a thinner and less structured biofilm. The central importance of the CepI/R system in biofilm formation was also confirmed in a subsequent study, in which three mutants were identified that had insertions in *yciR*, *suhB* and *yciL*. As these mutants showed reduced levels of AHLs, it was suggested that their biofilm defect is due to impaired expression of CepI/R (Huber *et al.*, 2001). In *B. cenocepacia* K56-2, the influence of the CepI/R and CciI/R QS systems on biofilm formation is rather complex: mutations in either *cepI* or *cepR* led to reduced amounts of biofilm; however, mutation of *cciI* or *cepI cciI* did not affect the ability to form a biofilm, while a *cciR* as well as a *cepR cciR* double mutant were deficient in biofilm formation (Tomlin *et al.*, 2005). As with strain H111, higher-level regulators appear to fine-tune QS-dependent biofim development of strain K56-2. The sensor kinase hybrid AtsR, which is an important regulator of type VI secretion system and virulence factor expression, was shown to affect the timing of AHL production in this organism. This effect is thought to be the reason for the biofilm defect observed with an *atsR* mutant (Aubert *et al.*, 2008).

The influence of QS on biofilm formation was confirmed for several *Burkholderia* species. A survey by Wopperer *et al.*, (2006) has shown that the large majority of Bcc strains, belonging to nine species, formed biofilms in an AHL-dependent manner (Wopperer *et al.*, 2006). In addition, mature biofilms of *B. cenocepacia* and *B. multivorans* were reduced upon treatment with QS-inhibitory compounds, reaffirming the importance of QS in the late stages of biofilm development (Brackman *et al.*, 2009). Likewise, it has been shown that biofilm formation of *B. pseudomallei* is dependent on AHL signalling (Gamage *et al.*, 2011).

In a recent study three AHL-regulated candidate gene clusters were tested for their role in biofilm formation of *B. cenocepacia* H111 (Inhülsen *et al.*, 2012). The three loci were (i) the *bclACB* lectin operon, (ii) the large surface protein *bapA* and its transporter and (iii) a gene cluster encoding a type I pilus. This analysis revealed that BapA plays a major role in biofilm formation on abiotic surfaces while inactivation of the type I pilus showed little effect both in a static microtitre dish-based biofilm assay and in flow-through cells. Inactivation of the *bclACB* lectin genes gave rise to biofilms with an aberrant structure (see above).

The BDSF-dependent QS system of *B. cenocepacia* was also shown to have a strong effect on biofilm formation (Deng *et al.*, 2012; Ryan *et al.*, 2009).

1.4 Interspecies and communication in mixed biofilms

Bcc strains and *P. aeruginosa* can form mixed biofilms in the lungs of CF patients. Given that both organisms control biofilm formation and expression of other virulence factors by QS, it appears likely that the two species are capable of communicating with each other. Initial evidence for interspecies signalling was reported by McKenney *et al.* (1995), who showed that the production of siderophores, lipases and proteases by *B. cepacia* can be stimulated by the addition of concentrated *P. aeruginosa* PAO1 culture supernatants. Circumstantial evidence for an interaction of the two pathogens was also obtained when the longitudinal AHL profiles of *P. aeruginosa* isolated from chronically infected CF patients were analysed (Geisenberger *et al.*, 2000). During the sampling period, two patients became co-infected with *B. cepacia*. While in one patient the observed AHL profile of *P. aeruginosa* isolates did not change, in the other case a dramatic reduction in the amount of AHL produced by *P. aeruginosa* was observed. Intriguingly, this reduction was only observed during the six-months period of co-infection. After clearance from *B. cepacia*, the *P. aeruginosa* AHL profile

was resumed (Geisenberger *et al.*, 2000). More direct evidence for AHL-mediated cross talk was obtained by the use of fluorescent biosensors, which were employed to visualize communication in mixed biofilms of *B. cenocepacia* H111 and clinical isolates of *P. aeruginosa* (Riedel *et al.*, 2001). In the two biofilm model systems that were used, artificial flow chambers and alginate beads introduced into the lungs of mice, a *cepI* mutant of *B. cenocepacia* was able to respond to the AHLs released by *P. aeruginosa*. Interestingly, the opposite, namely *P. aeruginosa* responding to *B. cenocepacia*, was not observed (Riedel *et al.*, 2001). In another study, however, it was demonstrated that *P. aeruginosa* and *B. cenocepacia* K56-2 were both able to utilize the heterologous signalling molecule (Lewenza *et al.*, 2002), suggesting that communication between the two organisms is strain-dependent.

Evidence is accumulating that *cis*-2-unsaturated fatty acids of the DFS family may also be used for interspecies communication (Ryan *et al.*, 2009). Many *Burkholderia* strains not only produce BDSF but some strains also synthesize DSF, the ancestral fatty acid signal isolated from Xcc, or other molecules of the DSF family (Deng *et al.*, 2010). In a recent study Twomey *et al.* (2012) examined the role of DSF and BDSF in the regulation of virulence and persistence of *P. aeruginosa* in the cystic fibrosis airways. *P. aeruginosa* does not produce BDSF or DSF but the structurally related compound *cis*-2-decenoic acid, which was shown to induce the dispersal of biofilms (Davies & Marques, 2009). Twomey *et al.* (2012) showed that the presence of DSF and BDSF in sputa of CF patients was correlated with the colonization of the patient with *B. cenocepacia* and/or *Stenotrophomonas maltophilia*, which is known to produce DSF (Fouhy *et al.*, 2007). In the presence of DSF, persistence of *P. aeruginosa* was increased in a CFTR knockout mouse model. Furthermore, polymyxin tolerance was enhanced when *P. aeruginosa* was grown as a biofilm in the presence of DSF on human airway epithelial cells (Twomey *et al.*, 2012). This study provided evidence that interspecies DSF-mediated bacterial interactions occur in the CF lung and it is tempting to speculate that this is also the case with BDSF, the main DSF family signal molecule produced by various *Burkholderia* species.

In addition to their roles in interspecies communication, DSF family signal molecules have also been implicated in interkingdom signalling. Many *cis*-2-unsaturated fatty acids, including DSF, BDSF and *cis,cis*-11-methyldodeca-2,5-dienoic acid, have been demonstrated to efficiently inhibit germ tube formation of the fungus *Candida albicans* (Boon *et al.*, 2008; Deng *et al.*, 2010).

2. Aim of the thesis

The aim of this PhD project was to identify and characterize novel components of the QS regulatory systems operating in *B. cenocepacia* H111.

When analyzing the $rpfF_{Bc}$ gene, I noticed a gene, which is situated directly downstream of $rpfF_{Bc}$ and is conserved in many members of the Bcc. This gene, the H111 homologue of BCAM0581, encodes a protein containing a PAS, a GGDEF and an EAL domain. The latter two domains are known to be involved in c-di-GMP turnover. In addition, this gene has been identified in a transposon mutant screen for strains defective in biofilm formation (Huber *et al.*, 2002). The genetic localization of BCAM0581 and the fact that it is conserved in different *Burkholderia* species and that the DSF QS system of *X. campestris* is known to exert its function via the second messenger c-di-GMP (He & Zhang, 2008), prompted me to investigate the BDSF-dependent QS system of *B. cenocepacia*. In the first part of this work, the focus lies on the characterization of that BCAM0581 homologue gene, referred to as *rpfR*.

In the course of this work, it became clear that, in analogy to the DSF signalling of *X. campestris*, the BDSF QS system functions through regulation of the levels of the second messenger c-di-GMP; however the underlying mechanisms were found to be fundamentally different. Therefore, in the second part of my thesis the influence of c-di-GMP on the physiology of *B. cenocepacia* H111 was studied.

3. Results

3.1 Identification and characterization of the BDSF receptor RpfR

3.1.1 Article I

Cis-2-dodecenoic acid receptor RpfR links quorum-sensing signal perception with regulation of virulence through cyclic dimeric guanosine monophosphate turnover

Deng Y*, Schmid N*, Wang C, Wang J, Pessi G, Wu D, Lee J, Aguilar C, Ahrens CH, Chang C, Song H, Eberl L, Zhang L-H

PNAS; 109(38):15479–84

* These authors contributed equally to the work

Own contribution:
Construction and phenotypic analyses of the following mutants and plasmids: rpfR, BCAM0227, pBBR-rpfR, pBBR-rpfR$_{AAL}$, pBBR-rpfR$_{GGAAF}$.
Writing and editing of the manuscript together with co-authors.

3.1.2 Unpublished results

The above mentioned paper was published in collaboration with the laboratory of Prof. Lian-Hui Zhang at the Institute of Molecular and Cell Biology in Singapore. However, work on the topic started independently, and it occurred that some of the work was performed in both laboratories in parallel. As the first outline of the publication was written by the Zhang lab, in cases where identical results were obtained in both labs, their data/graphs were included in the manuscripts. I nevertheless include here some of my own results, as an independent confirmation of the published results and also to emphasise that, even though most of the graphs published are from the Zhang lab, Y.D. and I equally contributed to the work.

In the transposon mutant screening seeking for mutants defective in biofilm formation, Huber *et al.* (2002) identified a gene that showed highest homology to *yciR*, a gene of unknown function, from *E. coli*. Analysis of the genetic surrounding revealed that this gene is situated adjacent to $rpfF_{Bc}$, the BDSF synthase gene. The analysis of the amino acid sequence of YciR further suggested an involvement of this protein in bis(3',5')-cyclic diguanylic acid (c-di-GMP) metabolism, as it contains (in addition to a PAS domain) a GGDEF domain and an EAL domain. Active GGDEF domains exhibit diguanylate cyclase activity, i.e. they catalyse the formation of c-di-GMP from two molecules of guanosine triphosphate, whereas EAL domains function as phosphodiesterases to cleave c-di-GMP into the linear dinucleotide pGpG. In Xcc, it is well known that DSF signalling is linked to c-di-GMP turnover (see Introduction). Three reasons made *yciR* an interesting research object: i) the genetic proximity of *yciR* to $rpfF_{Bc}$, ii) the involvement of both proteins in biofilm formation and iii) the already existing link of the DSF signal molecule and c-di-GMP metabolism.

As YciR turned out to be the BDSF receptor in the course of this work, it is hereafter referred to as RpfR (or *rpfR*).

3.1.2.1 An *rpfR* mutant phenocopies an $rpfF_{Bc}$ mutant

As starting point to study the function of *rpfR*, a defined knock-out mutant in this gene as well as in $rpfF_{Bc}$ was generated. Complementation was achieved by expressing *rpfR* from a

plasmid and by supplementing the growth medium with synthetic BDSF, respectively. The strains were subjected to various phenotypic tests.

Strikingly, the *rpfF*$_{Bc}$ mutant and the *rpfR* mutant were both defective in biofilm formation, proteolytic activity, swarming motility and swimming motility when compared to the wild type, as was previously observed with the Tn*5* insertion mutant m17 (Figure 2; Huber *et al.*, 2002). Interestingly, the *rpfR* mutant could not be restored by the addition of BDSF in the swarming assay. These results indicate that RpfR is involved in BDSF signalling.

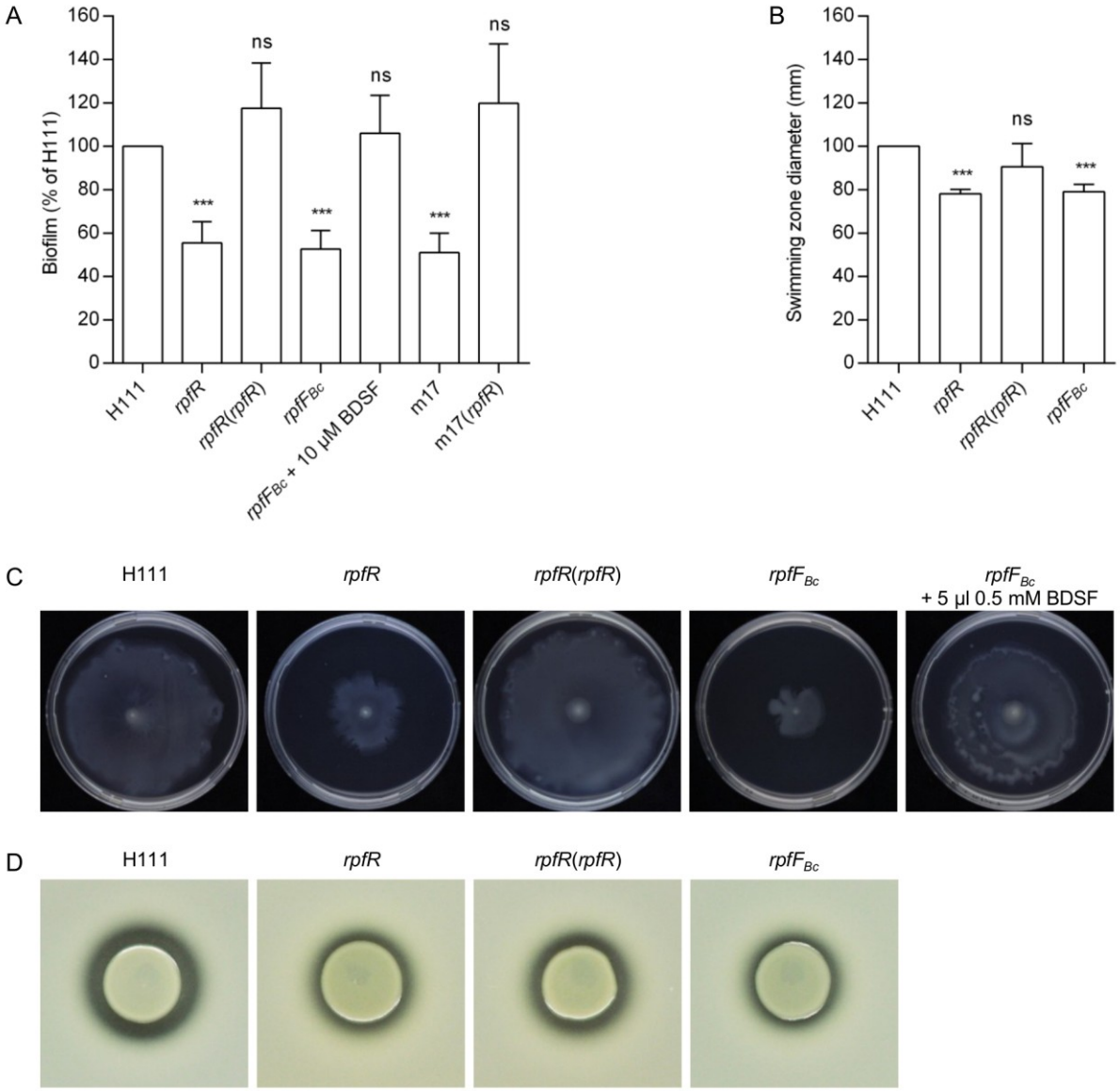

Figure 2. Phenotypes of an *rpfR* and an *rpfF*$_{Bc}$ mutant. (A) Biofilm formation. (B) Swimming motility. (C) Swarming motility. (D) Protease activity. Errorbars indicate SD, n≥3. ***: P<0.001, ns: not significant, one-way ANOVA with Tukey's post test, compared to H111. Pictures: representative results.

3.1.2.2 RpfR reveals diguanylate cyclase and phosphodiesterase activity

The amino acid sequence of RpfR revealed that it contains a PAS, a GGDEF and a EAL domain, a combination that is by far the most common architecture of c-di-GMP metabolizing enzymes (Römling *et al.*, 2013). Vice versa, PAS domains (Per-Arnt-*S*im) are very often associated with GGDEF and EAL domains (Henry & Crosson, 2011). PAS domains are found in bacteria, archaea and eukaryots and are defined by a characteristic fold composed of an anti-parallel, five-stranded β-sheet with a 2-1-5-4-3 strand order. Intervening α-helices form a pocket in which ligand binding occurs (Henry & Crosson, 2011). Predictions on what ligand a particular PAS domain is able to bind are very difficult, as the primary sequences of PAS domain differ greatly and so do the substance classes of already described PAS domain ligands.
The synthesis of c-di-GMP is catalysed by a homodimer of GGDEF domains. In the GGDEF (or GGEEF) signature motif, the first two (Gly) residues are involved in GTP binding, while the third and the fourth residues (Glu and Asp/Glu) are involved in metal ion coordination. Approximately half of the GGDEF domains also contain an I-site (inhibition site) shortly upstream of the signature motif. These four residues (RxxD) are responsible for allosteric product inhibition. RpfR of *B. cenocepacia* H111, however, does not contain such an I-site.
Unlike GGDEF domains, EAL domains retain their phosphodiesterase activity as monomers, even though the majority of characterized EAL domain proteins act as dimer. Degradation of c-di-GMP is achieved by hydrolysis of an ester bond to yield the linear dinucleotide pGpG. (Schmidt *et al.*, 2005).From the domain analysis of RpfR, it was not predictable whether RpfR possesses c-di-GMP synthesizing or degrading activity. To address this issue, RpfR was expressed heterologously, purified, and its enzymatic activity was measured. As controls, variant proteins with a mutation of either the GGDEF or the EAL motif were constructed and characterized along with the native RpfR. In addition, the intracellular levels of c-di-GMP of the wild type and various mutant strains were determined.

Incubation of the wild-type protein with GTP or with c-di-GMP resulted in complete conversion of the substrate to pGpG. This indicates that RpfR possesses diguanylate cyclase as well as phosphodiesterase activity *in vitro* (Figure 3 FG). In contrast, the two variant proteins are deficient in one of these activities. Rpf_{RAAL} is able to synthesize c-di-GMP from GTP but fails to degrade it whereas $RpfR_{GGAAF}$ is unable to synthesize c-di-GMP from GTP but its phosphodiesterase activity is still intact (Figure 3 BC). Interestingly, incubation of RpfR or the mutant proteins with 10 µM BDSF in addition to the substrates did not abolish

either activity (Figure 3 DEFG). However, it abolishes to the formation of an additional product when GTP is used as substrate (Figure 3 DF). This product elutes in the FPLC profile shortly before c-di-GMP and at different elution times from all the substrates testes. The nature of this compound remained unclear.

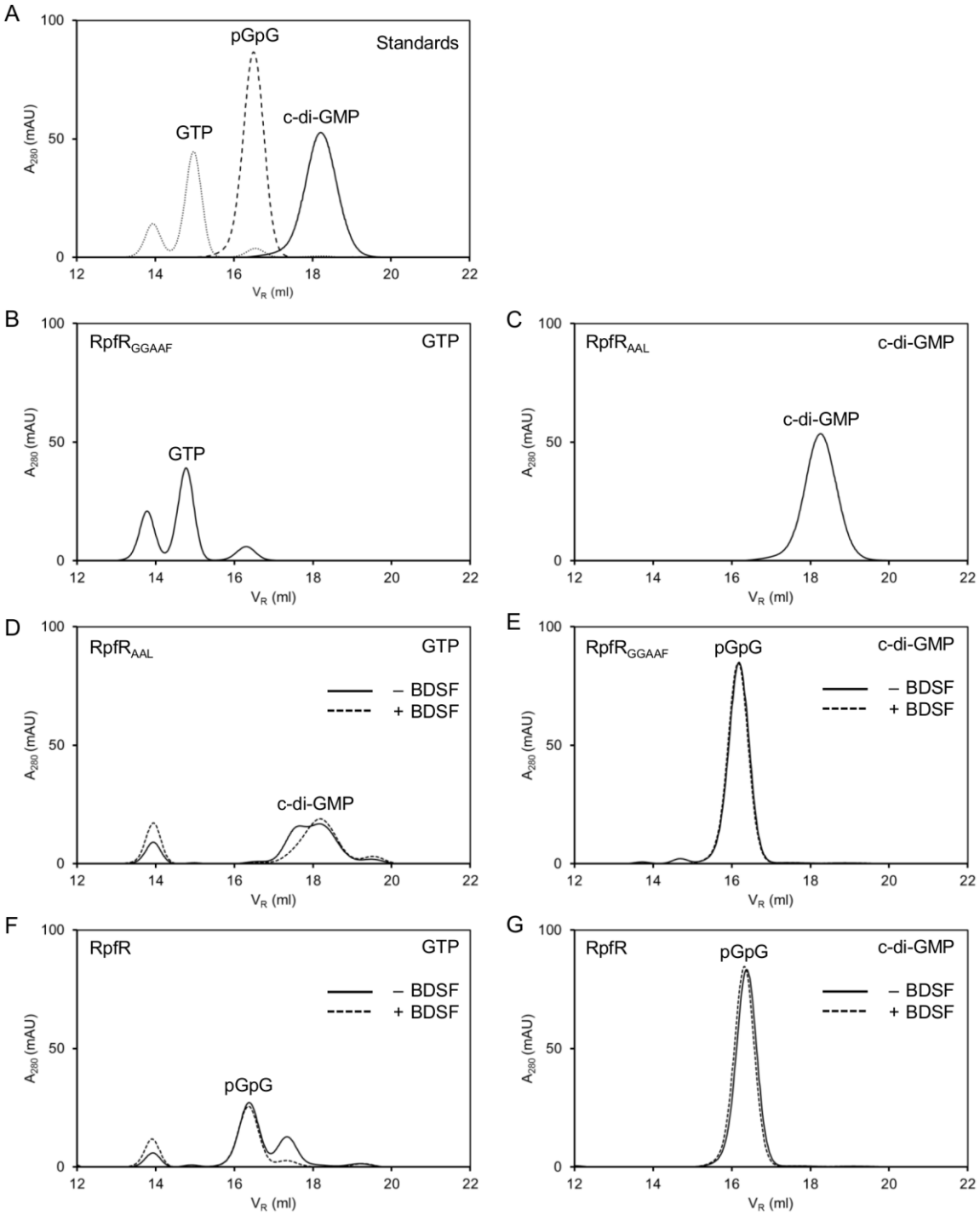

Figure 3. RpfR di-guanylate cyclase and phosphodiesterase activities. (A) FPLC analysis of the standards GTP, pGpG and c-di-GMP (A). The mutant proteins RpfR$_{GGAAF}$ and RpfR$_{AAL}$ are unable to utilize GTP (B) and c-di-GMP as substrates (C), respectively. RpfR$_{AAL}$ converts GTP to c-di-GMP (D) and RpfR$_{GGAAF}$ degrades c-di-GMP to pGpG (E). The wild-type RpfR shows complete conversion of GTP (F) or c-di-GMP (G) to pGpG.

These data show that RpfR is capable of synthesizing and degrading c-di-GMP. I next tested whether this is also true in the *in vivo* situation. To this end, the intracellular level of c-di-GMP in different genetic backgrounds was determined.

Mutation of *rpfR* increased the intracellular c-di-GMP concentration and, consistently with other phenotypic assays, mutation of $rpfF_{Bc}$ had the same effect. So *in vivo*, the phosphodiesterase activity of RpfR is predominant (see (Deng *et al.*, 2012)). Addition of 10 µM BDSF to the $rpfF_{Bc}$ mutant rescued the intracellular c-di-GMP levels to wild type (Figure 4 A).

Expression of RpfR from a plasmid did not lead to complementation of the *rpfR* mutant in this assay (cf. Deng *et al.*, 2012). Instead of lowered c-di-GMP level, this strain exhibit increased levels relative to the mutant. However, these results have to be interpreted with caution, as the variation between the 8 biological replicates was considerably high. Interestingly, when additional BDSF was provided to the culture medium, complementation was still not reached, but the overall values and the deviation between the replicates were much smaller (Figure 4 B). As a consequence, we examined strains that express RpfR or its variants with either an inactivated EAL or GGDEF domain from a plasmid in an *rpfR*-negative background. When mutating the GGDEF domain the levels of c-di-GMP dropped below the levels of the complemented *rpfR* mutant to about the level of the *rpfR* mutant. On the contrary, the protein with an inactivated EAL domain led to an extremely high level of c-di-GMP. Interestingly, supplementation of the growth medium with an excess of BDSF substantially lowered the c-di-GMP level of this strain, whereas the addition of extra BDSF to the GGDEF domain mutant did not lead to an altered c-di-GMP level. This finding suggests that BDSF has, additionally to its effect on phosphodiesterase activity, also an effect on c-di-GMP synthesis.

In summary, these experiments suggest that RpfR not only exhibits phosphodiesterase activity but is also capable of synthesizing c-di-GMP with both reactions being influenced by BDSF.

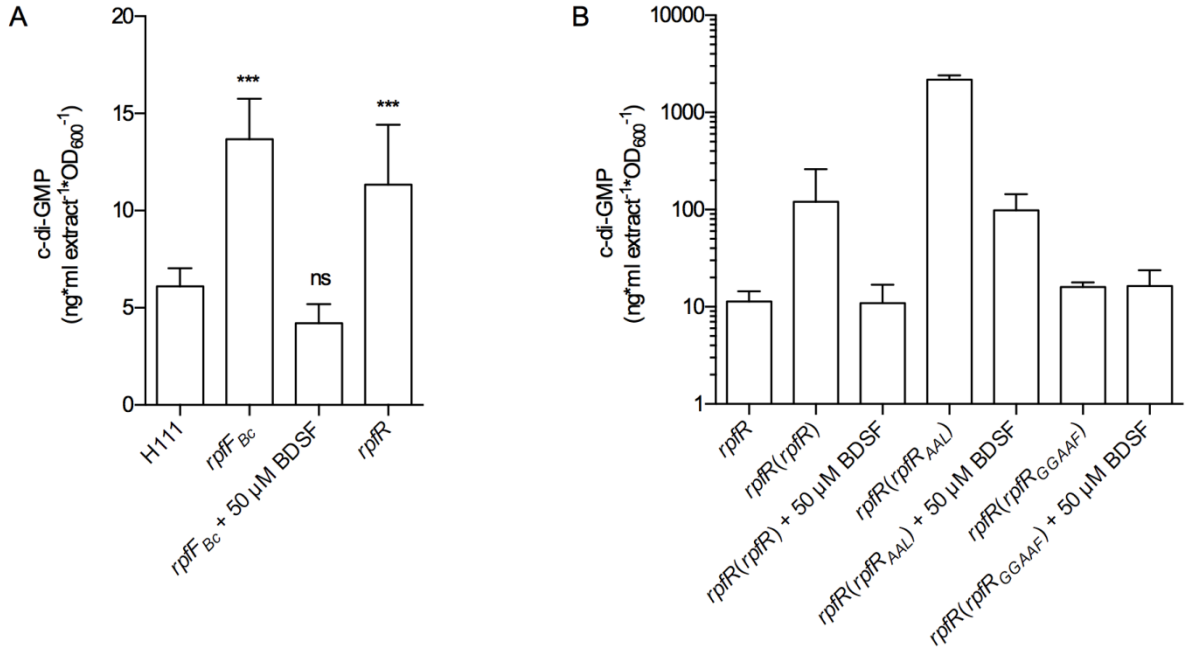

Figure 4. Quantification of c-di-GMP in different strains. (A) Intracellular c-di-GMP levels are elevated in an *rpfR* and an *rpfF*$_{Bc}$ mutant. Errorbars indicate SD, n≥3. ***: P<0.001, ns: not significant, two tailed t-test, compared to wild type. (B) Influence of the expression of RpfR or its variants from a plasmid in an *rpfR* mutant background. Errorbars indicate SD, n≥3. No post testing was applied as SD differs significantly between the groups.

3.1.2.3 RpfR negatively influences biofilm formation at the liquid-air interphase and on solid surfaces

Elevated c-di-GMP levels in bacteria are generally associated with the transition from motility to sessility and is therefore considered to be a main regulator of biofilm formation (e.g. reviewed in (Römling *et al.*, 2013)). In *B. cenocepacia* H111, however, the opposite was observed for submerged biofilms (i.e. biofilms formed at the solid-liquid interphase). Mutation of *rpfR* or $rpfF_{Bc}$ led to an increased intracellular c-di-GMP level (Figure 4 A) and concomitantly to impaired biofilm formation (Figure 2A). We wondered if the same is true for other biofilm models such as pellicle formation (i.e. the biofilm formed at the liquid-air interphase) or the wrinkly colony phenotype on agar plates.

Testing different media revealed that NYG broth or agar plates worked best to assess both phenotypes, whereas LB or minimal medium were clearly less suitable (data not shown). To evaluate pellicle formation, the strains were incubated in NYG broth at room temperature, until an opaque layer at the liquid-air interphase had formed. In case of *B. cenocepacia* H111 strains this took about seven days. The wild type then had formed a fragile pellicle that easily detached from the walls of the tubes, as seen in Figure 5. In marked contrast, the *rpfR* mutant formed a thick and very robust pellicle that remained intact after tilting of the tube. The same was also observed for the $rpfF_{Bc}$ mutant. Genetic complementation of the *rpfR* mutant reduced the strength and the thickness of the pellicle to wild-type levels. Interestingly, when the *rpfR* deficient strain was complemented with an RpfR protein with an EAL to AAL mutation, the pellicle formed is as robust as the pellicle of the *rpfR* mutant alone. In contrast, expressing a mutant RpfR, in which the GGDEF motive was changed to GGAAF in the *rpfR* deficient background, resulted in a wild-type pellicle.

The formation of a wrinkly morphotype on NYG plates as indication for biofilm formation on solid surfaces required a temperature shift from 37°C to ambient temperature and therefore plates with spotted bacteria were first incubated at 37°C, before shifting to room temperature. As shown in Figure 5, *B. cenocepacia* H111 wild type did not form this type of biofilm, i.e. the colony morphology was smooth. In marked contrast, an *rpfR* mutant exhibited a pronounced wrinkly colony morphology and consistent with previous results where the $rpfF_{Bc}$ mutant phenocopied the *rpfR* mutant, also the BDSF synthase mutant was wrinkly. Complementation of the *rpfR* mutant restored the wild-type appearance, but expression of the mutated version of RpfR with either the GGDEF or the EAL domain inactivated in the *rpfR* mutant background, failed to do so. For rpfR(rpfR$_{GGAAF}$) this is an interesting observation, as this phenotype is the only one where the mutated RpfR protein with intact PAS and EAL

domain, but no GGDEF domain, did not complement the mutant. For pellicle formation (Figure 5) as well as (submerged) biofilm formation, protease activity and swarming motility (see Figure S3 from (Deng *et al.*, 2012)), the presence of an intact GGDEF domain was not required.

Figure 5 also shows that a *bapA* mutant formed a wild-type pellicle and colony morphology. This indicates that, in contrast to the submerged biofilm formed at the solid-liquid interphase, BapA is neither essential for pellicle formation nor for the wrinkly colony morphotype.

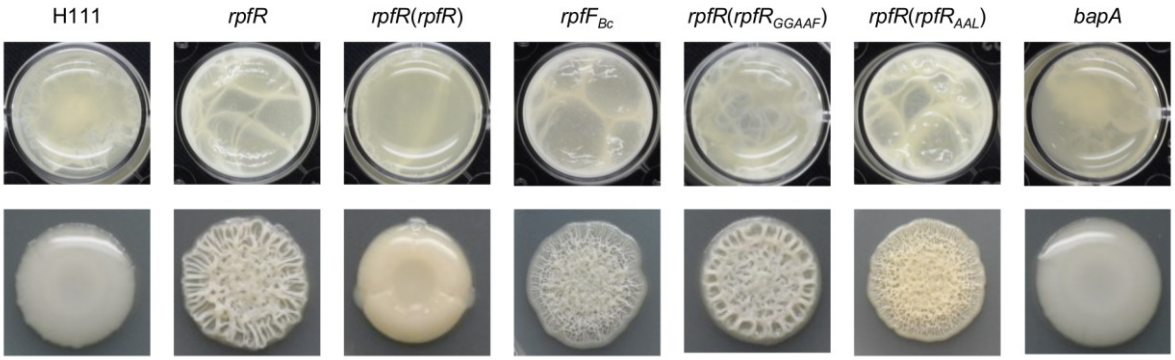

Figure 5. Pellicle formation and colony morphology is affected by *rpfR*. Top row: Pellicle formation. The mutants *rpfR*, *rpfF*$_{Bc}$ and *rpfR*(*rpfR*$_{AAL}$) form a thick, robust pellicle, whereas the wild type (H111), the complemented *rpfR* mutant, the mutant *rpfR* provided *in trans* with *rpfR*$_{GGAAF}$ and the *bapA* mutant form a thin, fragile pellicle. Note the scattered versus focused light reflexes within the different wells. Bottom row: Colony morphology. *B. cenocepacia* H111 wild type, the complemented *rpfR* mutant and the *bapA* mutant form smooth colonies. In contrast, the mutants *rpfR*, *rpfF*$_{Bc}$ and *rpfR*(*rpfR*$_{AAL}$) and *rpfR*(*rpfR*$_{GGAAF}$) show a wrinkly colony morphology.

3.2 QS in *B. cenocepacia* H111: an entangled web of BDSF, AHL and c-di-GMP signalling

3.2.1 Article II

The AHL- and BDSF-dependent quorum sensing systems control specific and overlapping sets of genes in Burkholderia cenocepacia H111

Schmid N*, Pessi G*, Deng Y*, Aguilar C, Carlier AL, Grunau A, Omasits U, Zhang L-H, Ahrens CH, Eberl L

PLoS ONE 7(11): e49966

* These authors contributed equally to the work

Own contribution:

Construction of the following mutants and plasmids: H111-rpfF$_{Bc}$, H111 ΔcepI-rpfF$_{Bc}$, pBBRcepI, pP_{cepI}-*lacZ,* pSHAFT-rpfF$_{Bc}$, pSU11Tp.

All phenotypic analyses (except BDSF quantification).

Line art in figure 5.

Writing of substantial parts of the manuscript.

Editing of the manuscript together with co-authors.

3.2.2 Unpublished results

3.2.2.1 The effect of BDSF on transcription of *cepI* is mediated through c-di-GMP

From the previous chapter it is clear that the AHL- and BDSF-dependent QS circuitries are not separated but interconnected. We found that the $rpfF_{Bc}$ mutant produces only 50% of the AHL levelof the wild type and that transcription of *cepI* is 3.2-fold down-regulated. A recent study in *B. cenocepacia* J2315 confirmed our findings (Udine *et al.*, 2013). However, the mechanism by which the BDSF system controls expression of *cepI* has not been examined in either study. An involvement of c-di-GMP, however, seemed likely due to the fact that many phenotypes such as biofilm formation, swarming motility and proteolytic activity are impaired in mutants deficient in the production of BDSF or AHLs or in the *rfpR* mutant.

To study the effect of different intracellular c-di-GMP levels on the physiology of *B. cenocepacia* H111, including AHL production, we employed constructs with the ability to alter the cellular c-di-GMP levels, including RpfR variants with mutated catalytical residues in the EAL or the GGDEF domain as described in 3.1.2.2. Another construct used to reduce c-di-GMP levels was provided by Prof. Urs Jenal, University of Basel. It constitutively expresses the phosphodiesterase gene PA5295 of *P. aeruginosa* from the *lac* promoter of pBBR1MCS-5. PA5295 (albeit expressed from a different plasmid) was shown to reduce c-di-GMP levels in *C. crescentus* below the detection limit. In order to test these three construct, we brought them into *B. cenocepacia* wild type via conjugative plasmid transfer and determined intracellular c-di-GMP levels. As shown in Figure 6, expressing $RpfR_{AAL}$ in the wild type led to dramatically increased c-di-GMP level from approx. 6 ng/ml extract to more than 2000 ng/ml extract, comparable to the level observed when this construct was present in the *rpfR* mutant background (Figure 4). On the other hand, $RpfR_{GGAAF}$ failed to reduce the c-di-GMP level, again similar to the effect seen in the *rpfR* mutant background. However, the *Pseudomonas* phosphodiesterase PA5295 was found to reduce the c-di-GMP level to around 25% of the wild-type level. Consequently, plasmid pBBR-$rpfR_{AAL}$ was used to study the effect of an increased intracellular c-di-GMP level and plasmid pBBR-PA5295 was used to examine the effect of a lowered intracellular c-di-GMP level in *B. cenocepacia* H111. Phenotypic analyses of these strains revealed that the c-di-GMP level influences AHL levels, likely through regulating expression of *cepI* (Figure 6 B). While the wild type harbouring the empty control plasmid or a plasmid expressing PA5295 are indistinguishable with respect to their AHL levels, the strain with dramatically increased c-di-GMP level, H111($rpfR_{AAL}$), was found to produce greatly reduced amounts of AHLs (Figure 6 B). Interestingly, the addition of

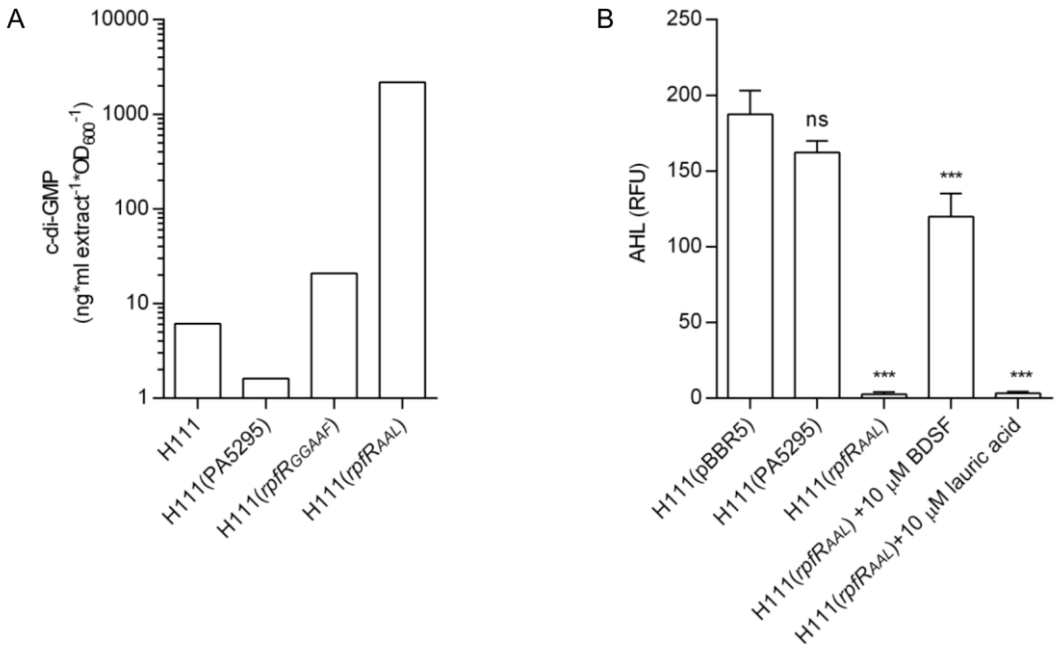

Figure 6. Effects of different constructs on the intracellular c-di-GMP concentration and on AHL level. (A) Modulating the intracellular c-di-GMP level of *B. cenocepacia* H111 by the presence of plasmids encoding GGDEF or EAL domain proteins. Data shown are from one representative experiment. (B) Production of AHL is reduced at high c-di-GMP levels. Errorbars indicate SD, n=3. ***: P<0.001, ns: not significant, one-way ANOVA with Tukey's post test, compared to the plasmid control pBBR5.

10 µM BDSF to the medium, but not of the fully saturated isomer lauric acid, led to an increase in AHL biosynthesis.

The nematocidal protein AidA is one of the most stringently AHL-regulated proteins of *B. cenocepacia* H111 (Huber *et al.*, 2004; Inhülsen *et al.*, 2012; Schmid *et al.*, 2012). I tested the expression of AidA as well as the *C. elegans* pathogenicity of the strains exhibiting different c-di-GMP levels (Figure 7). Western blot analysis demonstrated that, consistently with AHL production, expression of AidA is greatly reduced when the c-di-GMP level is increased. Supplementation of the growth medium with 200 nM AHL led to a partial restoration of AidA expression in H111(*rpfR*$_{AAL}$), indicating that the high c-di-GMP concentration directly interferes with AHL production rather than with AidA expression (Figure 7A). Due to the reduced expression of AidA, the pathogenicity of *B. cenocepacia* H111 with increased c-di-GMP level was completely attenuated in the *C. elegans* infection model. While with the wild type and the strain with reduced c-di-GMP levels, H111(PA5295) on plate, all nematodes died within 120 hours, however the nematodes showed no sign of sickness and propagated when fed on the high c-di-GMP strain H111*(rpfR*$_{AAL}$*)* (Figure 7B).

Taken together, these results suggest that the suppressing role of the BDSF on the AHL-dependent QS system is mediated through modulation of the intracellular c-di-GMP level.

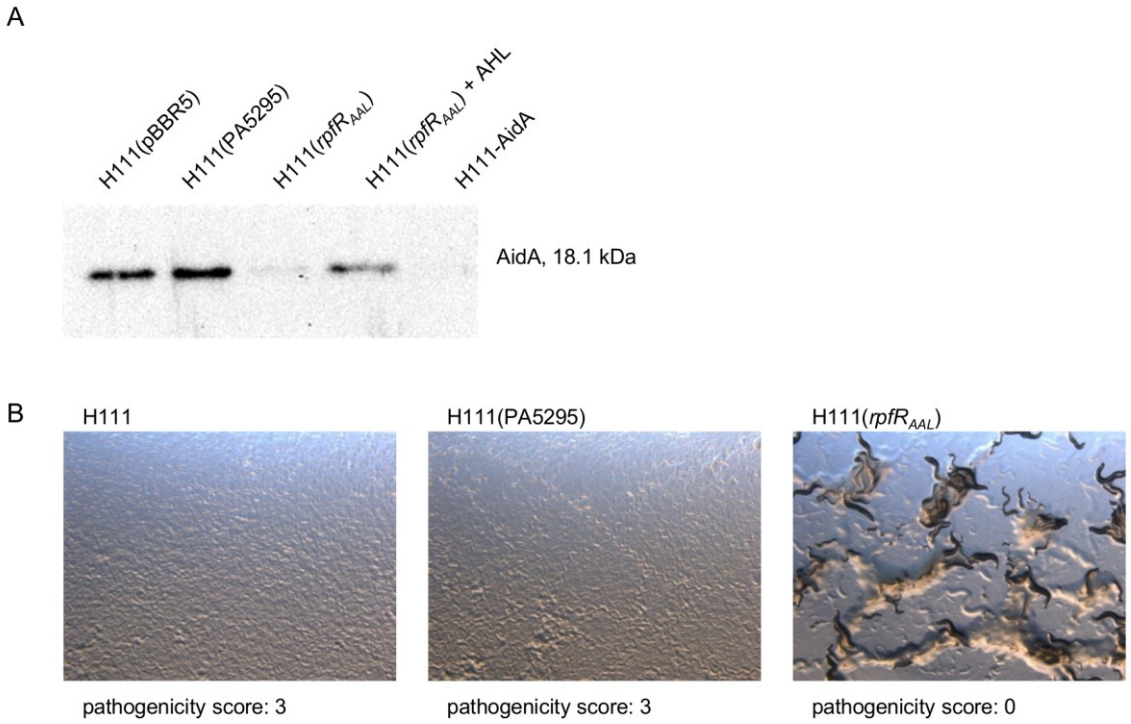

Figure 7. Reduced AidA expression and attenuated virulence when the intracellular c-di-GMP level is increased. (A) Western blot analysis of AidA expression in different strains. H111-AidA: *aidA* mutant (negative control). (B) Pathogenicity of strains against the nematode *C. elegans*. Pictures of a representative experiment were taken after 120 h incubation of *C. elegans* on NGM plates seeded with the respective *B. cenocepacia* strain. Pathogenicity scores were calculated from three independent experiments.

3.2.2.2 Changes in gene expression upon altered c-di-GMP levels

To further test the influence of different c-di-GMP level on gene expression in *B. cenocepacia* H111, the transcriptomes of strains with artificially increased or decreased c-di-GMP level was compared to the one of the wild-type strain. To this end, RNA-Seq of H111(pBBR5), H111(PA5295) and H111*(rpfR$_{AAL}$)* grown to late exponential growth phase was performed. Two independent replicates were performed for each strain. On the very same cultures that were used for the transcriptome analysis, c-di-GMP was quantified and confirmed the changes of c-di-GMP levels as described above in the wild type expressing RpfR$_{AAL}$ and c-di-GMP concentrations below the detection limit in the strain that expresses the *P. aeruginosa* phosphodiesterase PA5295 (Figure 8)

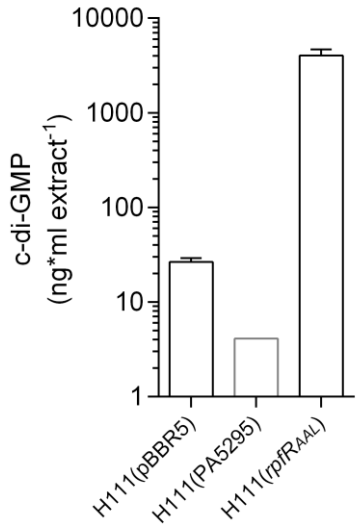

Figure 8. Quantification of c-di-GMP on the cultures used for transcriptome analysis. Average values from three technical replicates. Errorbars indicate SD of three technical replicates. The c-di-GMP concentration of H111(PA5295) was below the level of detection (< 4.12 ng/ml).

The percentage of mapped reads varied between 19% and 32%. This is considerably higher than what was obtained previously with a different methodology (Schmid *et al.*, 2012) and is in the range of what Peano and coworkers have recently reported (Peano *et al.*, 2013). A technical summary of the experiment is compiled in Table 2.

Analysis of the data revealed that the fold change calculation based on RPKM (reads per kilobase of transcript per million mapped reads) values for a given gene varied considerably between the two experiments. The reason for this is unknown. The frequency distribution of the RPKM values showed no major deviations, as depicted in Figure 9.

As normalization of RNA-Seq data is essential in order to account for the presence of systematic variation between and within samples (e.g. different library sizes, genes specific effects related to GC content or gene length) as well as differences in library composition (e.g.

presence of high-count genes, large numbers of 0 counts), unique gene counts were analysed with the statistic package DESeq2 (Anders & Huber, 2010). This methodology was shown to be particularly robust to different library sizes and different library compositions and was therefore applied (Dillies *et al.*, 2012).

With this procedure, we found a total of 113 genes to be significantly differentially expressed greater or equal 3-fold in at least one comparison, i.e. when the wild-type strain was compared to either the strain with increased or decreased c-di-GMP levels or when the strain with low c-di-GMP concentration was compared to the strain with high c-di-GMP concentrations (Table 3). This corresponds to 1.56 % of the total 7258 protein coding genes of *B. cenocepacia* H111. From those 113 genes, 15 show an up-regulation upon increasing the intracellular c-di-GMP concentration (from wild-type level to high c-di-GMP concentrations or from low c-di-GMP levels to high c-di-GMP concentration) and 98 genes show a down-regulation when c-di-GMP concentrations were increased in the cell.

Table 2. Technical summary of the RNA-Seq analysis.

	Sample	total number of reads	unique reads
Experiment 1	H111(pBBR5)	10 741 113	32% (3 459 293)
	H111(PA5295)	14 189 380	19% (2 755 271)
	H111(*rpfR*$_{AAL}$)	11 324 979	27% (3 002 172)
Experiment 2	H111(pBBR5)	5 484 288	24% (1 302 614)
	H111(PA5295)	6 162 915	21% (1 317 438)
	H111(*rpfR*$_{AAL}$)	7 883 458	19% (1 512 969)

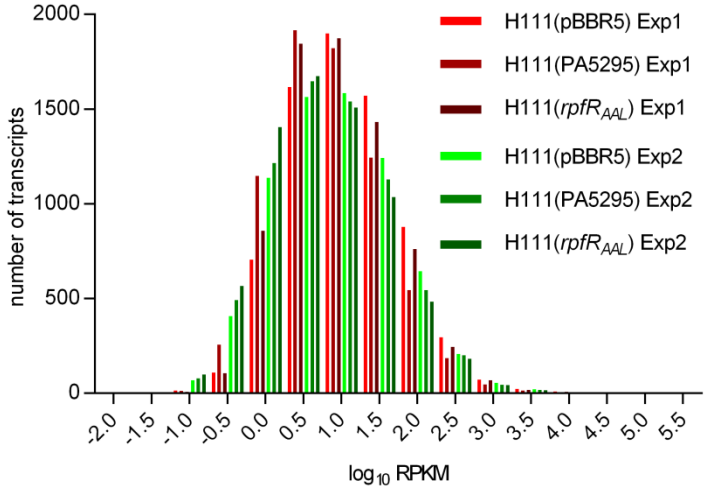

Figure 9. Frequency distribution of log-transformed RPKM values revealed no major deviation between the two sets of experiments.

Table 3. Genes regulated upon altered c-di-GMP level. 113 genes found to be ≥ 3 fold up- or down-regulated in *B. cenocepacia* H111 wild type compared to a strain with high (WT vs HIGH) or low (WT vs LOW) c-di-GMP levels or when the strain with reduced c-di-GMP concentration is compared to the strain with increased c-di-GMP concentrations (LOW vs HIGH).

Protein ID [a]	Locus Tag [a,b]	ids J2315 orthologs [c]	description	gene name [d]	WT vs HIGH [e]	WT vs LOW [e]	LOW vs HIGH [e]
CCE46507	I35_0315	BCAL0408	Phenylacetic acid degradation protein PaaN2,ring-opening aldehyde dehydrogenase	*paaZ*	2.45	-1.29	**3.41**
CCE46508	I35_0316	BCAL0407	Beta-ketoadipyl CoA thiolase	*paaJ*	2.42	-1.35	**3.76**
CCE46509	I35_0317	BCAL0406	Phenylacetate degradation enoyl-CoA hydratase PaaA	*paaG*	2.10	-1.33	**3.13**
CCE46510	I35_0318	BCAL0405	Phenylacetic acid degradation protein PaaD,thioesterase	*paaI*	1.93	-1.37	**3.39**
CCE46511	I35_0319	BCAL0404	Phenylacetate-coenzyme A ligase PaaF	*paaK1*	1.84	-1.49	**3.22**
CCE46549	I35_0357	BCAL0368	Cold shock protein CspA		**-3.48**	-1.19	-2.78
CCE46560	I35_0368	**BCAL0358**	Puromycin-sensitive aminopeptidase		**3.83**	-1.53	**7.07**
CCE46575	I35_0383	BCAL0343	Uncharacterized protein ImpD	*bscL*	**4.12**	-1.29	**4.89**
CCE46704	I35_0514	BCAM0201	Inner membrane component of tripartite multidrug resistance system		-1.14	**-3.42**	**4.47**
CCE46705	I35_0515	**BCAM0200**	Membrane fusion component of tripartite multidrug resistance system		-1.20	**-3.48**	**4.60**
CCE46706	I35_0516	BCAM0199	Outer membrane component of tripartite multidrug resistance system		1.23	**-3.54**	**5.94**
CCE46708	I35_0518	**BCAM0197**	transcriptional regulator, LysR family		**6.07**	-2.58	**16.79**
CCE46709	**I35_0519**	**BCAM0196**	hypothetical protein I35_0519		**5.39**	-1.78	**7.71**
CCE46710	**I35_0520**	**BCAM0195**	Peptide synthetase		**8.10**	-2.20	**19.25**
CCE46711	**I35_0521**	**BCAM0194**	hypothetical protein I35_0521		**6.44**	-1.82	**12.38**
CCE46712	**I35_0522**	**BCAM0193**	hypothetical protein I35_0522		**7.31**	-2.11	**21.98**
CCE46713	**I35_0523**	**BCAM0192**	Probable remnant of a transposase protein		**7.39**	-1.80	**9.28**
CCE46715	**I35_0525**	**BCAM0191**	Enterobactin synthetase component F		**6.38**	-1.45	**9.76**
CCE46716	**I35_0526**	**BCAM0190**	Omega-amino acid--pyruvate aminotransferase		**5.74**	-1.49	**7.20**
CCE46717	I35_0527	**BCAM0189**	transcriptional regulator, AraC family	*cepS*	**4.99**	-1.58	**8.58**
CCE46718	I35_0528	**BCAM0188**	transcriptional activator protein solR	*cepR2*	2.14	-1.51	**3.40**
CCE46720	**I35_0530**	**BCAM0186**	Photopexin A	*bclA*	**3.82**	-1.75	**11.70**
CCE46721	**I35_0531**	**BCAM0185**	Photopexin A	*bclC*	1.84	-2.35	**4.48**
CCE46722	**I35_0532**	**BCAM0184**	Photopexin A	*bclB*	2.73	-1.64	**4.94**
CCE46874	I35_0684	**BCAM0028**	hypothetical protein I35_0684		**3.47**	-1.19	**4.33**
CCE46998	I35_0810	BCAM1712	3-hydroxybutyryl-CoA dehydrogenase precursor	*paaH*	2.84	-1.56	**5.16**
CCE46999	I35_0811	BCAM1711	Enoyl-CoA hydratase/isomerase	*paaK2*	2.74	-1.43	**4.79**
CCE47473	I35_1285	BCAM1293	ABC-type dipeptide transport system, periplasmic component		**3.51**	-1.08	**3.40**
CCE47584	I35_1396	**BCAM1187**	Ferrichrome-iron receptor		**-5.00**	1.14	**-5.34**
CCE47886	I35_1708	BCAL1525	Flp pilus assembly protein, pilin Flp		**3.71**	1.12	2.95
CCE48206	I35_2037	**BCAL0510**	hypothetical protein I35_2037		**3.07**	-1.16	**3.86**
CCE48385	I35_2218	**BCAM0634**	hypothetical protein I35_2218		4.30	-1.53	**6.71**
CCE48386	I35_2219	**BCAM0633**	Leucyl aminopeptidase (aminopeptidase T)		**5.00**	-1.35	**8.18**
CCE48387	I35_2220	BCAM0632	Acetyltransferase, GNAT family		2.94	-1.38	**4.42**
CCE48446	**I35_2279**	BCAM0581	Enoyl-CoA hydratase/carnithine racemase	*rpfFBc*	**-4.83**	-1.05	**-4.94**
CCE48447	I35_2280	BCAM0580	PAS/PAC domain protein	*rpfR*	**-8.24**	1.06	**-9.80**
CCE48524	I35_2357	BCAM0505	putative osmotically inducible protein Y		2.49	-1.12	**3.24**
CCE48634	I35_2467	**BCAM0393**	Beta-lactamase class D		2.99	-1.06	**3.05**

Table 3 (continued)

Protein ID [a]	Locus Tag [a,b]	ids J2315 orthologs [c]	description	gene name [d]	WT vs HIGH [e]	WT vs LOW [e]	LOW vs HIGH [e]
CCE48637	I35_2470	BCAM0390	Alkaline phosphatase		-1.83	2.19	**-4.61**
CCE48638	I35_2471	BCAM0389	Alkaline phosphatase		-1.81	2.46	**-5.07**
CCE48728	**I35_2565**	**BCAM1010**	UTP--glucose-1-phosphate uridylyltransferase	*gtaB*	2.05	-2.83	**7.20**
CCE48821	I35_2658	BCAM0922	Xanthomonapepsin precursor		**3.72**	1.07	**3.59**
CCE48876	**I35_2721**	BCAM0810	Ortho-halobenzoate 1,2-dioxygenase alpha-ISP protein OhbB		**3.08**	1.01	**3.86**
CCE48910	I35_2755	**BCAM0776**	cAMP-binding proteins-catabolite gene activator and regulatory subunit of cAMP-dependent protein kinases		-1.73	1.79	**-3.21**
CCE48911	I35_2756	BCAM0775	Glutathione S-transferase		-2.22	1.50	**-3.52**
CCE49126	I35_2975	BCAM2008	TPR repeat protein, SEL1 subfamily		-2.90	1.13	**-3.07**
CCE49127	I35_2976	**BCAM2007**	Ferrichrome-iron receptor		**-3.32**	-1.05	**-3.39**
CCE49240	I35_3092	BCAL0116	Ferrichrome-iron receptor		**-3.09**	-1.43	-1.99
CCE49427	**I35_3284**	BCAL1974	Glycogen debranching enzyme		**3.35**	-1.38	**4.97**
CCE49631	I35_3490		hypothetical protein I35_3490		**3.26**	-1.05	**3.54**
CCE49632	I35_3491	**BCAL2786**	selenium binding protein, putative		**3.68**	-1.00	**3.47**
CCE49799	I35_3662	BCAL3002	hypothetical protein I35_3662		-1.76	1.72	**-3.60**
CCE49804	I35_3667	**BCAL3006**	Cold shock protein CspG		**5.18**	1.19	**3.93**
CCE50228	I35_4105	BCAM2378	Xaa-Pro dipeptidyl-peptidase		**4.74**	1.16	**3.79**
CCE50229	**I35_4106**	BCAM2377	hypothetical protein I35_4106		**4.09**	1.12	**4.06**
CCE50321	**I35_4199**	**BCAM1871**	hypothetical protein I35_4199		**9.53**	-1.31	**11.45**
CCE50322	I35_4200	**BCAM1870**	N-acyl-L-homoserine lactone synthetase RhlL	*cepI*	**10.06**	-1.11	**12.46**
CCE50475	**I35_4355**	BCAL2352	Carbonic anhydrase		**5.37**	-1.17	**4.72**
CCE50476	**I35_4356**	**BCAL2353**	Sulfate permease		**6.71**	-1.27	**6.49**
CCE50758	I35_4641	BCAL1819	Oxidoreductase (flavoprotein)		-1.29	2.42	**-7.89**
CCE50759	I35_4642	BCAL1819	Oxidoreductase (flavoprotein)		-1.37	2.24	**-5.50**
CCE50760	I35_4643	BCAL1818	Zn-dependent hydrolases, including glyoxylases		-1.04	2.60	**-4.50**
CCE50892	**I35_4778**	BCAM0859	Tyrosine-protein kinase Wzc	*bceF*	1.50	-1.74	**3.31**
CCE50893	**I35_4779**	BCAM0858	Polysaccharide export lipoprotein Wza	*bceE*	1.75	-1.77	**3.61**
CCE50895	**I35_4781**	BCAM0855	UDP-glucose dehydrogenase	*bceB*	1.62	-1.99	**3.82**
CCE50898	**I35_4784**	BCAM0854	Mannose-1-phosphate guanylyltransferase (GDP) ; Mannose-6-phosphate isomerase	*bceA*	2.26	-1.64	**3.61**
CCE50899	**I35_4785**	BCAM0853	transposase and inactivated derivatives		2.44	-1.49	**3.44**
CCE50917	**I35_4803**	**BCAM0835**	transcriptional regulator, AraC family		1.57	-2.33	**4.17**
CCE51106	I35_4997	BCAL0216	Phenylacetate-CoA oxygenase, PaaG subunit	*paaA*	2.15	-1.64	**3.92**
CCE51108	I35_4999	BCAL0214	Phenylacetate-CoA oxygenase, PaaI subunit	*paaC*	1.88	-1.54	**3.05**
CCE51188	I35_5082	BCAL3212	Membrane protein		**3.08**	-1.09	**3.22**
CCE51244	**I35_5138**	**BCAL3285**	Flavohemoprotein (Hemoglobin-like protein) (Flavohemoglobin) (Nitric oxide dioxygenase)		2.68	-1.66	**5.74**
CCE51321	I35_5216	BCAL1386	putative lipase in cluster with Phosphatidate cytidylyltransferase		**3.98**	1.00	**3.79**
CCE51401	I35_5296	BCAL1294	VgrG protein		**3.61**	-1.08	**3.80**
CCE51940	I35_5851	BCAS0153	putative membrane protein YPO1990		**3.48**	-1.22	**4.35**
CCE51941	I35_5852	BCAS0154	putative inner membrane protein		2.92	-1.06	**3.15**
CCE51942	I35_5853	BCAS0155	putative cytoplasmic protein		**3.43**	-1.09	**3.82**
CCE52069	I35_5980	BCAS0256	Outer membrane protein (porin)		**4.24**	1.12	**3.11**
CCE52072	I35_5983	**BCAS0258**	transcriptional regulator, GntR family		**3.32**	1.24	2.65
CCE52107	**I35_6018**	BCAS0291	Periplasmic binding protein		**8.02**	-1.58	**11.01**
CCE52108	**I35_6019**	**BCAS0292**	AidA	*aidA'*	**4.78**	-1.28	**7.23**

Table 3 (continued)

Protein ID [a]	Locus Tag [a,b]	ids J2315 orthologs [c]	description	gene name [d]	WT vs HIGH [e]	WT vs LOW [e]	LOW vs HIGH [e]
CCE52109	**I35_6020**	**BCAS0293**	AidA	*aidA*	**3.63**	-1.20	**4.27**
CCE52251	I35_6162	**BCAS0409**	Bacillolysin precursor	*zmpA*	**3.60**	-1.09	**3.89**
CCE52252	I35_6163		hypothetical protein I35_6163		**3.22**	-1.03	**3.62**
CCE52939	**I35_6869**	**BCAM2308**	Bacterial leucyl aminopeptidase		**3.77**	-1.18	**5.91**
CCE52940	**I35_6870**	**BCAM2307**	Bacillolysin	*zmpB*	**4.58**	-1.16	**6.41**
CCE52992	I35_6922	BCAM2254	VgrG protein		**3.82**	-1.23	**4.63**
CCE52993	I35_6923	BCAM2253A	hypothetical protein I35_6923		**3.49**	-1.25	**4.26**
CCE52994	I35_6924	BCAM2253	Rhs-family protein		2.41	-1.25	**3.25**
CCE52997	I35_6927	BCAM2251	Extracellular ligand-binding receptor		**4.37**	1.19	**3.91**
CCE53012	I35_6942	BCAM2236	transcriptional regulator		1.55	-1.84	**5.84**
CCE53013	**I35_6943**	BCAM2235	Isochorismate synthase [pyochelin] siderophore	*pchA*	1.81	-1.49	**12.30**
CCE53014	I35_6944	BCAM2234	Isochorismate pyruvate-lyase [pyochelin] siderophore	*pchB*	1.61	-1.56	**7.30**
CCE53015	**I35_6945**	BCAM2233	Pyochelin biosynthetic protein PchC, putative thioesterase	*pchC*	1.77	-2.03	**11.74**
CCE53016	**I35_6946**	BCAM2232	2,3-dihydroxybenzoate-AMP ligase [pyochelin] siderophore	*pchD*	1.56	-1.51	**6.53**
CCE53019	**I35_6949**	BCAM2230	Dihydroaeruginoate synthetase PchE, non-ribosomal peptide synthetase modules	*pchE*	1.36	-1.65	**5.53**
CCE53020	**I35_6950**		Pyochelin synthetase PchF, non-ribosomal peptide synthetase module	*pchF*	1.66	-1.41	**9.98**
CCE53021	**I35_6951**	**BCAM2227**	Pyochelin biosynthetic protein PchG,oxidoreductase (NAD-binding)	*pchG*	2.05	-1.86	**14.45**
CCE53022	**I35_6952**	BCAM2226	ABC efflux pump, fused inner membrane and ATPase subunits in pyochelin gene cluster	*pchH*	1.18	-1.43	**4.21**
CCE53023	**I35_6953**	BCAM2225	ABC efflux pump, fused inner membrane and ATPase subunits in pyochelin gene cluster	*pchI*	1.41	-1.50	**7.15**
CCE53024	**I35_6954**	BCAM2224	Outer membrane receptor for ferric-pyochelin FptA	*ftpA*	1.32	-1.60	**6.57**
CCE53026	I35_6956	BCAM2222	putative iron-regulated membrane protein FtpC in pyochelin gene cluster	*ftpC*	1.48	-1.76	**7.17**
CCE53027	I35_6957	BCAM2221	Inner-membrane permease FptX, ferripyochelin	*ftpX*	1.21	-2.20	**3.53**
CCE53104	I35_7035		hypothetical protein I35_7035		2.36	-1.37	**3.22**
CCE53105	I35_7036	BCAM2154	hypothetical protein I35_7036		**3.20**	-1.11	**3.57**
CCE53117	**I35_7048**	**BCAM2143**	Large repetitive protein (BapA)	*bapA*	**11.89**	-1.35	**11.75**
CCE53118	**I35_7049**	**BCAM2142**	Agglutination protein		3.82	-1.90	**6.29**
CCE53119	**I35_7050**	**BCAM2141**	LktB		2.97	-1.85	**5.27**
CCE53120	**I35_7051**	**BCAM2140**	HlyD family secretion protein		2.68	-1.77	**4.91**
CCE53259	I35_7190	BCAM2057	Type III secretion inner membrane channel protein (LcrD,HrcV,EscV,SsaV)	*bcscV*	2.13	-1.45	**3.12**
CCE53261	I35_7192	BCAM2055	General secretion pathway protein D	*bcscC*	2.00	-1.61	**4.05**
CCE53262	I35_7193	BCAM2054	hypothetical protein I35_7193	*bcscD*	1.52	-1.88	**3.16**
CCE53263	I35_7194	BCAM2053	hypothetical protein I35_7194		1.77	-1.75	**3.36**

[a]: Nomenclature according to GenBank file CAFQ01000000.
[b]: Genes with I35 locus tags in bold have previously been identified with transcriptomics to be regulated by the RpfF/R QS system (doi: 10.1371/journal.pone.0049966).
[c]: Orthologous genes in bold have previously been identified to be ≥2 fold up- or down-regulated by the CepI/R system in *B. cenocepacia* H111 (doi: 10.1002/mbo3.24).
[d]: Gene names according to nomenclature of B. cencocepacia J2315, doi: 10.1007/s10534-006-9065-4, doi: 10.1128/JB.00481-08 or doi: 10.1002/mbo3.24-.
[e]: Significant foldchanges (with adjusted p-values <0.1) in bold.

Among the highly c-di-GMP regulated genes was *cepI*. Increasing c-di-GMP levels from wild type or even from depleted c-di-GMP concentrations led to a down-regulation of this gene of 10.06 and 12.46 fold, respectively. Consequently, from the 113 genes found to be regulated by c-di-GMP, 42 genes (including *cepI*) have previously been shown to be regulated by the CepI/R QS system of *B. cenocepacia* H111 (Inhülsen *et al.*, 2012). Out of those, 24 genes were previously also described to be within the BDSF stimulon (Schmid *et al.*, 2012), (Figure 10). The genes identified in all the three transcriptomes include *aidA*, the zinc metalloprotease *zmpB*, the lectins *bclACB*, *bapA* and the adjacent type I secretion system (BCAM2140-42), and a cluster encoding for a non-ribosomal peptide synthetase (BCAM0190-96) (see Table 3, Figure 10).

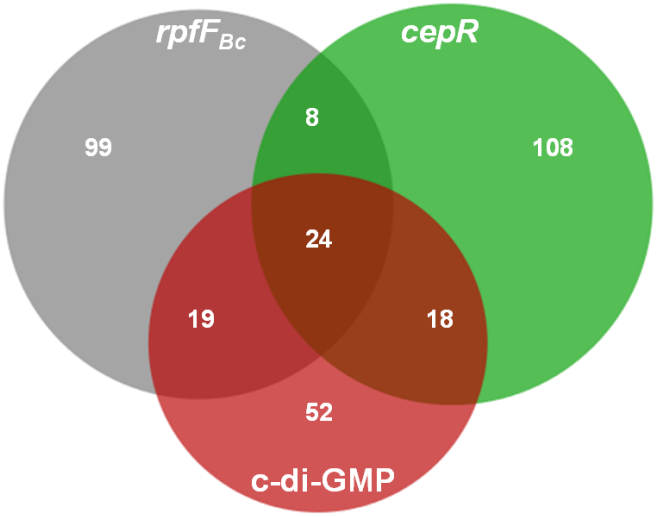

Figure 10. Venn diagram displaying the overlap between the c-di-GMP, the BDSF and the AHL stimulon. The BDSF and the AHL stimulons were assessed by transciptome analysis of the BDSF synthase RpfF$_{Bc}$ and of the AHL receptor CepR, respectively, as published in Inhülsen *et al.*, 2012 and Schmid *et al.* 2013.

The core genes of the BDSF QS system, *rpfF$_{Bc}$* and *rpfR*, were found to be up-regulated under high c-di-GMP conditions. This is an artefact, as in the strain with highly increased c-di-GMP concentration, *rpfR* (with a point mutation) and parts of *rpfF$_{Bc}$* were expressed from the plasmid used to elevate the intracellular c-di-GMP level and therefore more mRNA transcripts were detected. When comparing H111(pBBR5) with H111(PA5295), the strains with unchanged and reduced c-di-GMP level, respectively, neither *rpfF$_{Bc}$* nor *rpfR* were up-regulated.

From the genes regulated by c-di-GMP, 37 % (43 genes) were also regulated by the RpfF/R QS system, as identified by analysing the transcriptome of an *rpfF$_{Bc}$* mutant (Schmid *et al.*, 2012). Among those 43 genes, 19 were not affected by the AHL-dependent QS system.

When comparing the strain with depleted intracellular c-di-GMP to the strain with highly increased c-di-GMP concentration, we found five genes of the two cepacian clusters (BCAM1004-10 and BCAM0854-64) to be down-regulated under high c-di-GMP conditions.

We have previously identified those genes as positively regulated by BDSF, so this is in agreement with the phosphodiesterase activity of the RpfR protein. Furthermore, 12 genes of the pyochelin synthesis cluster (BCAM2221-35) were found to be up-regulated when H111(PA5295) was compared to H111*(rpfR$_{AAL}$)*, of which 8 have previously also been shown to be BDSF-regulated and one gene seems to be jointly regulated by the AHL- and the BDSF-QS systems.

Some interesting gene clusters were also identified among the exclusively c-di-GMP regulated genes. Most prominently, genes within three clusters encoding for the phenylacetate catabolic pathway were identified. All with the exception of *paaF* were more than twofold down-regulated in the strain with high c-di-GMP concentrations compared to the strain with depleted intracellular c-di-GMP pool (Figure 11).

Additionally, we identified four genes (BCAM2053-55, BCAM2057) of the type III secretion system cluster (BCAM2045-57) among the exclusively c-di-GMP regulated genes in Table 3.

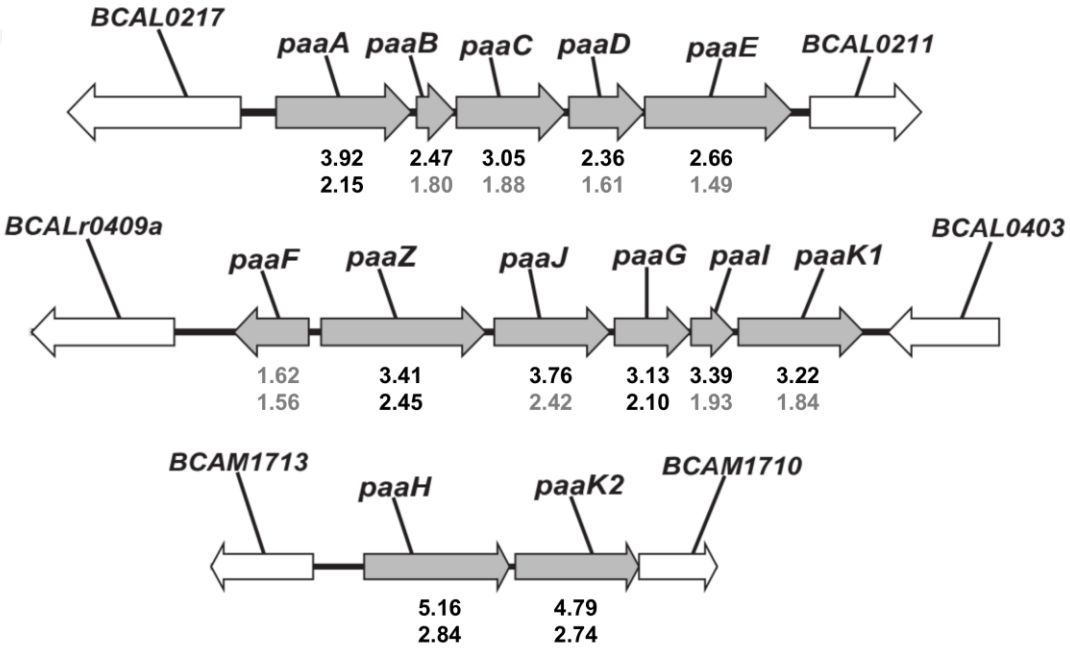

Figure 11. Genetic organization and regulation by c-di-GMP of the phenylacetate degrading gene clusters. Numbers below the genes indicate the fold changes found when H111(PA5295) compared to H111(*rpfR$_{AAL}$*) (LOW vs HIGH, top line) and when H111(pBBR) was compared to H111(*rpfR$_{AAL}$*) (WT vs HIGH, bottom line), respectively. Numbers in gray depict fold changes with an adjusted p-value >0.1. Adapted from (Law *et al.*, 2008).

3.2.2.3 Alteration of the c-di-GMP level in *B. cenocepacia* H111 affects physiology and virulence

I next investigated the phenotypic changes associated with increasing or decreasing intracellular c-di-GMP concentration in *B. cenocepacia* H111. In Figure 12, the results of the phenotypic tests are summarized. H111*(rpfR$_{AAL}$)* with a highly increased c-di-GMP level completely lacked proteolytic activity and the ability to swarm and was deficient in siderophore production, biofilm formation and swimming motility, whereas colony morphology on NYG plates and pellicle formation were not affected. Interestingly, *B. cenocepacia* H111 with depleted intracellular c-di-GMP formed no pellicle at all. This strain also possessed increased swimming and swarming motility. Other phenotypes, however, were not affected. Some of the mentioned phenotypes nicely confirmed the RNA-Seq data. We see reduced expression of a protease, of *bapA* and its type 1 secretion system and of the pyochelin cluster. Other phenotypes such as swarming or swimming motility lack explanation, as the flagella genes or genes responsible for rhamnolipid biosynthesis appear to be unaffected by altered c-di-GMP levels. The defect in bacterial motility is not apparent from the gene list in Table 3, however looking at individual genes that take part in flagella driven motility revealed that a gene encoding for the stator protein MotB (CCE48908, BCAM0778) shows a moderate, but statistically significant down-regulation (foldchange 2.58, adjusted p-value: 0.04) in H111(*rpfR$_{AAL}$)* when compared to H111(PA5295). Mutants of *motB* are still able to assemble the flagellum; however its rotation is impaired and renders the bacteria non-motile (Blair *et al.*, 1991). Reduced expression of *motB* in *B. cenocepacia* may result in a less flagellated and therefore less motile strain.

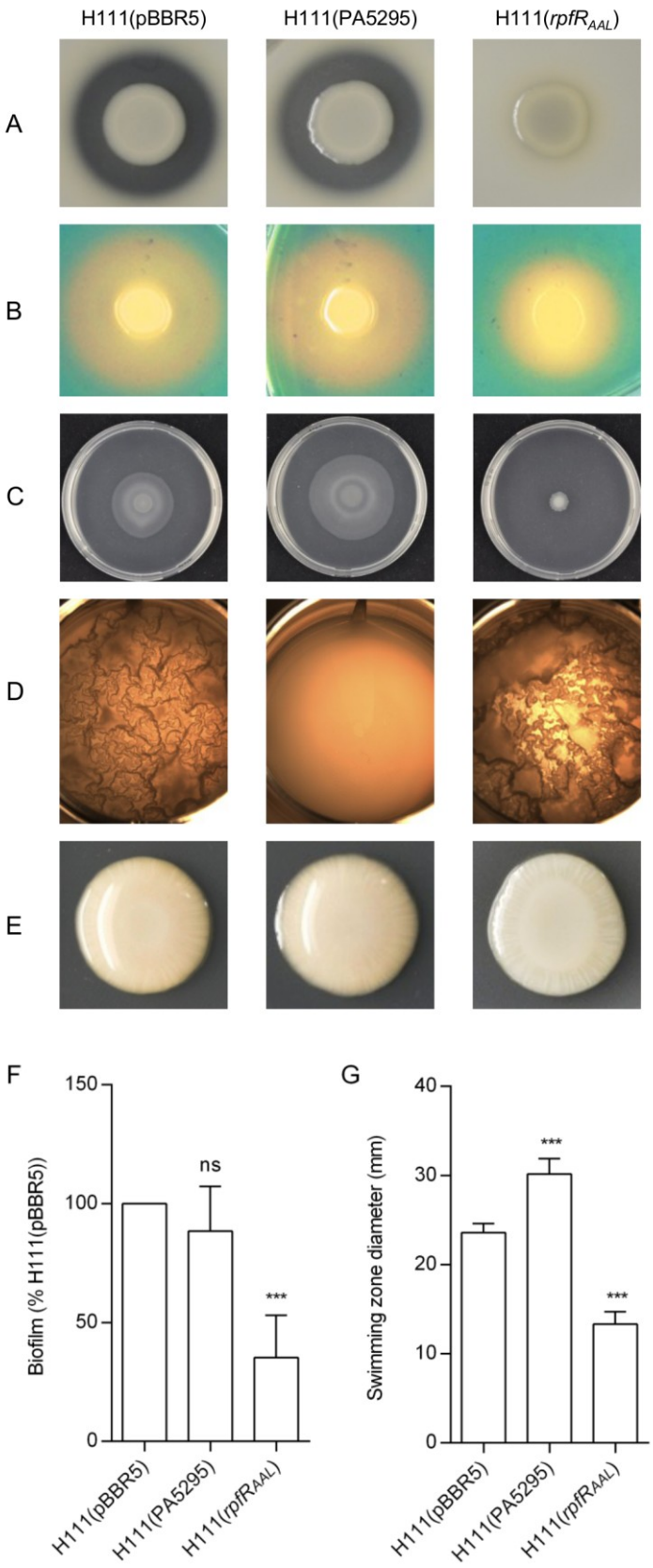

Figure 12. Phenotypes of strains with altered c-di-GMP levels. (A) Proteolytic activity on skim milk plates. (B) Siderophore production on CAS plates. (C) Swarming motility on NB 0.5% agar plates. (D) Pellicle formation in NYG broth. (E) Colony morphology on NYG plates. (F) Biofilm formation in ABC medium. (G) Swimming motility on NB 0.3% agar plates. Pictures shown are representatives from at least three independent replicates. Errorbars indicate SD, n≥6. ***: P<0.001, ns: not significant, One-way ANOVA with Tukey's post test, compared to H111(pBBR5).

Some of the above mentioned phenotypes are known to be important virulence factors in *B. cenocepacia* strains. Therefore we tested the strains with altered c-di-GMP level for their pathogenicity in the *C. elegans* and the *G. mellonella* infection models. H111*(rpfR$_{AAL}$)* completely lost its ability to kill *C. elegans*, whereas H111(PA5295) was indistinguishable from the wild type (see Figure 7). The reason for the attenuation of the high c-di-GMP strain is likely the down-regulation of *aidA*. The transcription of *aidA* was strongly reduced and expression of AidA was also found to be abolished by Western blot analysis (Figure 7). Other factors, such as the phenylacetate catabolism genes or the type 3 secretion system may also play a role but were not further followed up.

In addition to *C. elegans*, the strains were also tested in the *G. mellonella* infection model. In this model AidA is not essential for pathogenicity and thus the contribution of the other virulence factors can be assessed (Uehlinger *et al.*, 2009). Interestingly, H111*(rpfR$_{AAL}$)* was also highly attenuated in this infection model. More than half of the larvae were dead 48 hours after infection with H111(pBBR5) or H111(PA5295), but most of them were still alive when infected with H111*(rpfR$_{AAL}$)*. This pattern was even more pronounced 72 hours post infection (Figure 13).

In summary, alteration of the intracellular c-di-GMP level led to marked changes in the physiology and virulence of *B. cenocepacia* H111.

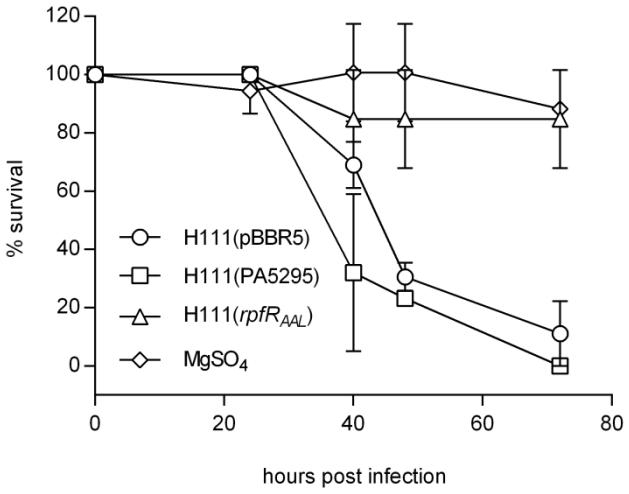

Figure 13. Increased c-di-GMP levels attenuate virulence in a *G. mellonella* infection model. Larvae of *G. mellonella* were infected with *Burkholderia* strains exhibiting wild-type (H111(pBBR)), decreased (H111(PA5295)) or increased (H111(*rpfR$_{AAL}$*)) c-di-GMP level and were scored for death or alive at the indicated time points post infection. $MgSO_4$: control (buffer only). Error bars indicate SD, n=3.

3.3 Identification of factors that link QS and biofilm formation

3.3.1 Identification of a putative BapA regulator

BapA (biofilm associated protein A) of *B. cenocepacia* H111 belongs to the family of large surface proteins that are usually secreted via a type I secretion system. Several members of this protein family have been shown to play a role in biofilm formation in different bacterial species. BapA has been shown to be of crucial importance for biofilm formation on abiotic surfaces, influencing the architecture as well as the biomass of the biofilm (Inhülsen *et al.*, 2012). The expression of BapA is regulated by both the AHL- and the BDSF-dependent QS system. However the downstream regulatory mechanism has not yet been elucidated. As there is no cep-box in the promoter region of *bapA*, a direct regulation by the CepR/AHL complex seems unlikely and the downstream effectors of the BDSF circuitry are currently unknown.

In an attempt to identify putative transcriptional regulators of *bapA*, a transposon mutant screening was performed. To this end, the *cepI* $rpfF_{Bc}$ double mutant of *B. cenocepacia* was mutagenized with a miniTn*5* transposon. Then, the P_{bapA}-*lacZ* promoter fusion was transferred into the triple mutant pool and clones that do no longer responded to the exogenous addition of BDSF with the development of an intense blue colour were further analysed. In total, 86000 colonies (see 4.3.10), were screened and 13 mutants with lowered β-galactosidase activity studied in further details.

Identification of the transposon insertion sites by arbitrary PCR revealed that in four of the mutants the *rpfR* gene had been inactivated (Table 4), providing a proof of concept of the screening procedure.

A gene encoding an IclR-family transcriptional regulator was found to be inactivated in three independent mutants. The archetype of this family of transcriptional regulators is IclR from *E. coli*, which regulates the *aceBAK* operon that encodes the enzymes responsible for the glyoxylate bypass (Maloy & Nunn, 1982). This shunt is induced upon growth on acetate or fatty acids as sole carbon source and it prevents the quantitative loss of the entering carbon as CO_2 in the tricarboxylic acid (TCA) cycle (Kornberg, 1966). IclR-family transcriptional regulators consist of an N-terminal helix-turn-helix motif responsible for DNA binding and a C-terminal effector binding domain. Interestingly, all the three transposon insertions were mapped to the C-terminal domain, between bases 555/556, 626/627 and 644/645 of 825 base pairs (see Appendix). The nature of the effectors of IclR-family transcriptional regulators is mostly unclear. Some described effectors are glyoxylate and puryvate of the *E. coli* IclR (Lorca *et al.*, 2007), 1-naphthol, 2,3-dihydroxynaphthalene, 4-nitrotoluene, benzonitrile and

indole of the *Pseudomonas putida* TtgV IclR (Guazzaroni *et al.*, 2005) and gamma-hydroxybutyrate and succinic semialdehyde of the *Agrobacterium tumefaciens* AttJ IclR (Chai *et al.*, 2007). The IclR family of transcriptional regulators includes repressors, activators and proteins with dual function, i.e. activating the transcription of some genes while suppressing the transcription of others (Molina-Henares *et al.*, 2006).

To confirm the finding of the screening and to further assess the role of the above identified IclR-family transcriptional regulator, a knock out mutant in this gene was generated, which was named *B. cenocepacia* H111-iclR. Firstly, the *bapA* promoter activity was determined in the wild type and the *iclR* mutant background. In agreement with the screening results, the promoter activity was significantly reduced in the mutant background, suggesting that IclR plays a role in controlling transcription of *bapA* (Figure 14A). As BapA is a key factor of biofilm formation in *B. cenocepacia* H111, the *iclR* mutant was tested for its ability to form biofilms. Similar to a *bapA* mutant, the *iclR* mutant was found to be highly impaired in biofilm formation (Figure 14B). It is interestingly to note that in *B. cenocepacia* J2315, a strain known to be a weak biofilm former (Wopperer *et al.*, 2006), *iclR* is annotated as pseudogene (BCAL1082), because it contains a nonsense mutation (ochre) after codon 46 (Holden *et al.*, 2009).

As several other phenotypes, such as swimming motility and proteolytic activity, were also found to be affected by a mutation in *iclR* (Results C. Aguilar), we mapped the IclR regulon by gene expression profiling using RNA-Seq. Preliminary data analysis revealed that IclR indeed regulates not only the expression of *bapA*, but also of other genes such as the lectin operon *bclACB*, the zinc-metalloprotease *zmpB*, genes from a cluster encoding for the siderophore ornibactin and others. So IclR seems to be a global transcription regulator rather than only regulating *bapA*.

Work of C. Aguilar in our group is currently ongoing to further unravel the nature of this transcriptional regulator and demonstrating its interaction with the *bapA* promoter region.

Table 4. Location of transposon insertions of *B. cenocepacia* mutants that no longer correspond to exogenous BDSF.

H111 RefSeq identifier [a]	J2315 orthologue	gene product	number of hits
I35_2157	BCAM0689	Methyl-accepting chemotaxis protein	2
I35_2280	BCAM0580	RpfR	4
I35_2281	BCAM0578	Similar to 5-oxoprolinase and methylhydantoinases A, B	1
I35_2284	BCAM0575	Transcriptional regulator, LysR family	1
I35_2307	BCAM0552	Transcriptional regulatory protein zraR	1
I35_2340	BCAM0525A	Chaperonin GroEL (HSP60 family)	1
I35_2423	BCAM0431	Glutathione S-transferase	1
I35_3394	BCAL1870	Transcription accessory protein (S1 RNA-binding domain)	1
I35_3981	no orthologue[b]	zinc-binding protein	1
I35_3982	BCAL3450	Mutator mutT protein	1
I35_4865	BCAL0960	Lipid A core - O-antigen ligase and related enzymes	1
I35_5431	BCAL1082[c]	Transcriptional regulator, IclR family	3
I35_6723	BCAL2117	ATP-dependent RNA helicase	1

[a]: Nomenclature according to GenBank file CAFQ01000000

[b]: intergenic region between BCAL3449 and BCAL3450

[c]: annotated as pseudogene

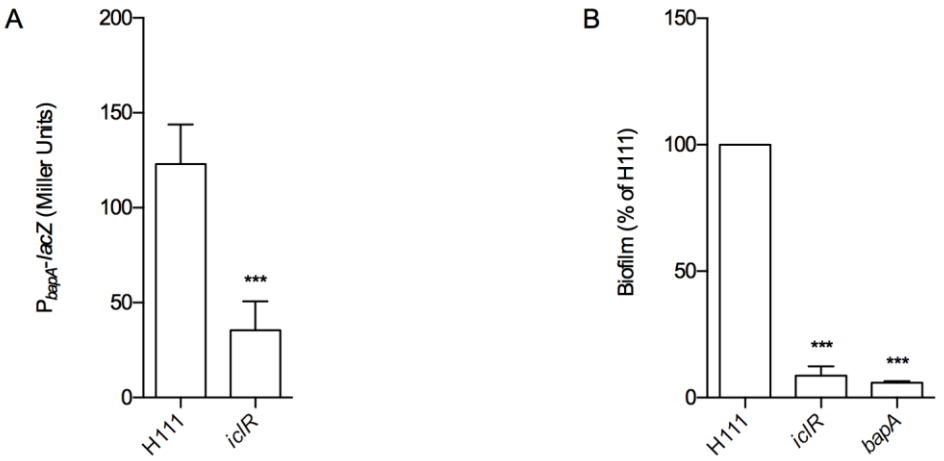

Figure 14. Promoter activity of *bapA* and biofilm formation is reduced in the *B. cenocepacia* H111 *iclR* mutant compared to the wild-type strain. (A) The promoter activity was measured in stationary phase. Error bars indicate SD, n=4. *** P<0.001, two-tailed t-test, compared to H111. (B) Biofilm formation in ABC minimal medium. Error bars indicate SD, n≥3. *** P<0.001, one-way ANOVA, Tukey's post test, compared to H111.

3.3.2 Rhamnolipids

Rhamnolipids (RHLs) are biosurfactants, produced by various species and consist of one or two rhamnose moieties and mainly one or two fatty acid chains of different length (varying from C8 to C16) linked to each other via an O-glycosidic linkage (Abdel-Mawgoud, Lepine *et al.* 2010). This biosurfactant has various functions in bacteria: it promotes the uptake and biodegradation of poorly soluble substrates, it acts as immune modulator and virulence factor, it has broad antimicrobial properties, it is crucial for surface motility and it is involved in biofilm development (Abdel-Mawgoud, Lepine *et al.* 2010). Furthermore, rhamnolipid production of *P. aeruginosa* (in which the RHLs have been extensively investigated) is QS-regulated. Two of the three genes necessary for RHL synthesis are organized in a gene cluster together with *rhlI* and *rhlR*, the *luxI/R* homologues of *P. aeruginosa* (Ochsner and Reiser 1995). Biosynthesis of rhamnolipids in *P. aeruginosa* involves three sequential steps: RhlA is involved in the synthesis of the fatty acid moiety, RhlB links the fatty acid moiety to a rhamnose molecule and RhlC adds an additional rhamnose moiety. In *B. cenocepacia* H111 we found homologs of *rhlA* (44% identity to PAO1 with tblastn), *rhlB* (48%) and *rhlC* (48%), albeit in a different genetic organization. The production of rhamnolipids in *Burkholderia* sp. has been reported for *B. plantarii* (Andra, Rademann *et al.* 2006), *B. pseudomallei* (Haussler, Nimtz *et al.* 1998), *B. thailandensis* (Dubeau, Deziel *et al.* 2009), *B. glumae* (Costa *et al.*, 2011) and *B. kururiensis* (Tavares *et al.*, 2013). As the biosynthesis of RHLs is tightly controlled by QS and as the RHLs are known to influence biofilm formation, they may link cell-cell communication and biofilm formation in *B. cenocepacia* H111.

To address this question, mutants in either *rhlA* or *rhlB* were generated. Rhamnolipid extraction and subsequent thin layer chromatography (TLC) revealed differences between the wild-type *B. cenocepacia* H111 and the two RHL deficient mutants (Figure 15). Furthermore, the two mutants were unable to swarm, but this defect could be restored by the addition of RHL extracts of *B. cenocepacia* H111 or *P. aeruginosa* PAO1 (Figure 17). However, there was no measurable difference between wild type and the two RHL-negative mutants in the surface tensions of culture supernatants or in biofilm formation, both tested in different media and at different time points (data not shown).

By means of promoter-fusion experiments, a possible regulation through the AHL-dependent QS system on transcription of *rhlA* was assessed. The promoter activities in the wild type and in the *cepR* mutant were indistinguishable (Figure 16), even though in a previous transcriptome analysis a slight effect of CepR on transcription of *rhlA* has been observed.

Transcriptome and proteome analyses of the BDSF synthase mutant *rpfF*$_{Bc}$ revealed no participation of this QS system in the regulation of the rhamnolipid genes. The data suggest that the transcription of the RHL synthase genes in *B. cenocepacia* H111 is not regulated by either of the two QS systems.

The *rhlA* mutant was also sent to Prof. M. Givskov's lab with the intention to test polymorphonuclear leukocyte toxicity. An initial experiment, in which *B. cenocepacia* H111 was tested for its ability to lyse red blood cells as an indication of RHL production, revealed only very low RHL activity for the wild type strain and no RHLs in the *rhlA* mutant (data not shown). This is in good agreement with the unchanged surface tension of the RHL mutants, but impedes testing of polymorphonuclear leukocyte toxicity.

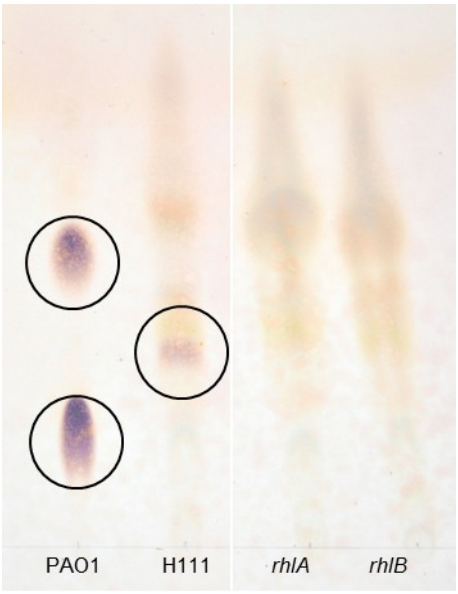

Figure 15. TLC analysis of rhamnolipid extracts of *P. aeruginosa* PAO1, the wild type *B. cenocepacia* H111, and the *rhlA* and *rhlB* mutant strains. 10 µl of extract was spotted for the *B. cenocepacia* strains and 1 µl for *P. aeruginosa*. Marked with circles are the stained rhamnolipid spots. The picture is processed (deletion of a lane).

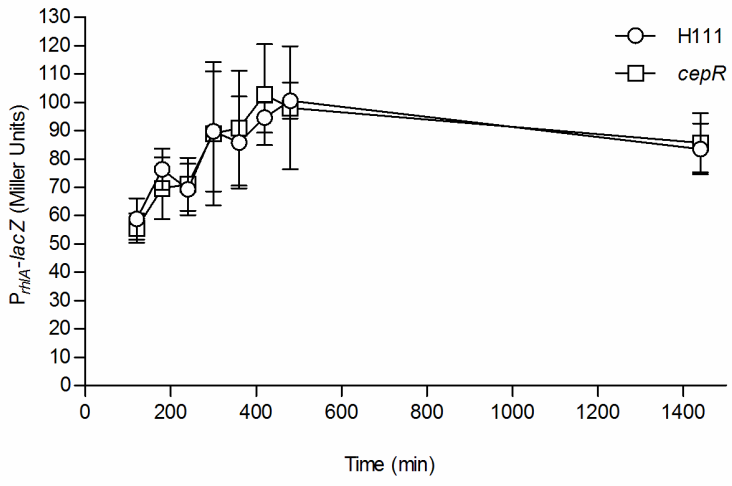

Figure 16. The expression of *rhlA* is not regulated by the AHL QS system. The *rhlA* promoter activitiy was assessed by means of transcriptional *lacZ* fusions in the H111 wild-type strain and in the *cepR* mutant over the time course of 24 hours. Error bars indicate SD, n=6.

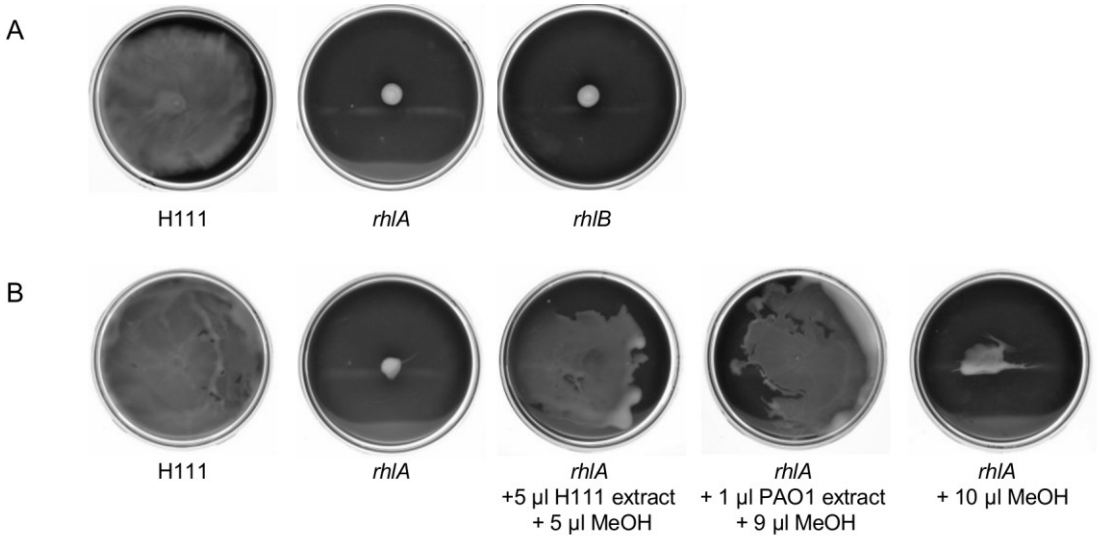

Figure 17. Swarming motility of the rhamnolipid mutants is abolished and can be complemented with rhamnolipid extracts. (A) Swarming motility of the wild type, the *rhlA* and the *rhlB* mutant. (B) Swarming complementation assay of the *rhlA* mutant with rhamnolipid extracts from *B. cenocepacia* H111 wild type or *P. aeruginosa* PAO1 and with methanol (MeOH) as solvent control.

4. Discussion

4.1 Identification and characterization of the BDSF receptor RpfR

The rational for this work was the observation that disruption of *rpfR* led to the same phenotypic changes as did disruption of the adjacent BDSF synthase $rpfF_{Bc}$. Reduced bacterial motility, decreased biofilm formation, compromised proteolytic activity, the ability to form strong pellicles and a wrinkly colony morphotype suggested that RpfR is a key component of the BDSF signalling pathway (Figure 2, Figure 5). In fact, we showed that BDSF binds to the PAS domain of the purified RpfR protein and causes an allosteric conformation change in the protein which in turn enhances c-di-GMP degradation conferred by the EAL domain. This is in good agreement with the observation that in an $rpfF_{Bc}$ mutant (and in the *rpfR* mutant) c-di-GMP levels are elevated (Figure 4 A). However, not all *in vitro* assays capture this fact. The analysis of the enzymatic activity of purified RpfR as described in 3.1.2.2 showed no effect of BDSF on the PDE activity. This may have been due to differences in methodology: for the far-UV circular dichroism analysis and enzymatic activity analysis of RpfR as published in Deng *et al.* (2012), the protein was pre-incubated with BDSF for one hour at room temperature prior to testing, whereas in the assay described in 3.1.2.2, BDSF was added immediately before testing. The latter analysis, however, revealed another interesting property of RpfR: it not only possesses PDE activity as described by Deng *et al.*, (2012), but showed that RpfR is able to synthesize c-di-GMP. The arrangement of GGDEF and EAL domains in tandem, i.e. on the same polypeptide chain, is found frequently. Römling and coworkers reported that 1/3 of all GGDEF domains and 2/3 of all EAL domains are arranged in tandems (Römling *et al.*, 2013). In most of the described enzymes with such an arrangement, however, one of the domains is inactive. Therefore, RpfR is one of the few exceptions with experimentally verified bifunctional activity. Another bifunctional enzyme is the recently described *Pseudomonas putida* PP2258 protein. This protein is important for motility on solid surfaces and both its EAL and GGDEF domains are functional (Österberg *et al.*, 2013). Recent work on ScrC from *Vibrio parahaemolyticus* also suggests an active role for both the EAL and the GGDEF domain of this protein. In the absence of the cognate S-signal (a novel signal molecule) the GGDEF domain elevates the intracellular c-di-GMP level and represses swarming, but upon the accumulation of the S-signal, the phosphodiesterase domain is activated and leads to differentiation into swarmer cells (Trimble & McCarter, 2011). Another bifunctional enzyme that modulates its activity depending on a signal is the

Rhodobacter sphaeroides protein BphG1, a bacteriophytochrome with a photosensory module linked to GGDEF-EAL output domains. BphG1 shows c-di-GMP specific PDE activity. Interestingly, BphG1 undergoes partial cleavage when expressed heterologously in *E. coli* and the truncated peptide lacking the EAL domain gains DGC activity, which is strongly activated by light. If the EAL domain is cleaved off in the native host is unclear (Tarutina *et al.*, 2006). In yet another GGDEF-EAL protein, the Lpl0329 protein from *Legionella pneumophila*, a phosphorylation-based switch modulates synthesis and turnover of c-di-GMP (Levet-Paulo *et al.*, 2011). The bifunctional enzyme MSDGC1 of *Mycobacterium smegmatis*, which is essential for long-term survival under nutrient starvation, consists of a GAF, a GGDEF and an EAL domain. It exhibits both enzymatic activities simultaneously *in vitro*, whereas the isolated domains or a truncated protein lacking the GAF domain are inactive (Bharati *et al.*, 2012).

Because BDSF was shown to stimulate the PDE activity of RpfR, the question arose if this signalling molecule also affects the synthesis of c-di-GMP. Hints for an influence of BDSF on the DGC activity of RpfR can be found in the *in vitro* as well as *in vivo* assays. Upon incubation of the wild-type RpfR or the mutated $RpfR_{AAL}$ with BDSF, the FPLC elution profile of the reaction products slightly changed in that an additional product was no longer observed (Figure 3). The nature of this product, however, is unclear, as none of the used substrates co-eluated. Another indication derived from analyses of the intracellular c-di-GMP level in the *rpfR* mutant expressing $RpfR_{AAL}$, i.e. a strain where only the cyclase activity of RpfR remained. When this strain was incubated with 50 µM BDSF, the intracellular c-di-GMP concentration dropped to only 4.5% of the concentration of the untreated control, suggesting that BDSF influences the cyclase activity of RpfR.

In trans expression of RpfR with an inactivated GGDEF domain was sufficient to complement pellicle formation, submerged biofilm formation, protease activity and swarming motility of the *rpfR* mutant. The latter three phenotypes could also be restored by the expression of RocR, a *P. aeruginosa* phosphodiesterase, (Deng *et al.*, 2012). However, wrinkly colony formation seems to be an exception as in this case the full length RpfR is required to complement the *rpfR* null-mutant phenotype.

The wrinkly colony morphotype was recently studied in more detail in *B. cenocepacia* H111 (Fazli *et al.*, 2011, 2012). In a transposon mutant screening, the transcription regulator BCAM1349 was identified as no longer responding to increased intracellular c-di-GMP

levels. BCAM1349 is a transcriptional regulator of the CRP/FNR superfamily and it comprises an N-terminal nucleotide binding domain that was shown to bind c-di-GMP and a C-terminal helix-turn-helix domain. The latter recognizes a sequence motif in the promoter region of a 12-gene cluster, BCAM1330-41, that encodes for an unknown exopolysaccharide. It was shown that over-expression of BCAM1349 leads to a wrinkly colony morphology and pellicle formation and promotes biofilm formation by increasing transcription of this gene cluster. Binding of BCAM1349 to the promoter region was shown to be enhanced by c-di-GMP. Interestingly, over-expression of the diguanylate cyclase YedQ from *E. coli* not only increased c-di-GMP levels but also resulted in the same phenotypical changes as over-expression of BCAM1349 (Fazli *et al.*, 2011). In contrast, increasing c-di-GMP levels by the aid of plasmid pBBR-rpfR$_{AAL}$ in the wild-type background did neither lead to the formation of wrinkly colonies or pellicles nor did we observe increased biofilm formation. The observed discrepancies may be due to additional c-di-GMP independent functions of RpfR (see below) and/or due to different media used. This most likely also explains why in our transcriptome analyses of *B. cenocepacia* H111 with highly increased c-di-GMP levels (Table 3) and of the *rpfR* mutant (our unpublished data) none of the genes of the above mentioned exopolysaccharide cluster was found to be differentially regulated. We used late exponential cultures grown in LB Lennox broth, whereas Fazli and coworkers used cultures grown for 48 hours on AB glucose plates (Fazli *et al.*, 2012).

Does an increased c-di-GMP level promote a sessile lifestyle and thus biofilm formation in *B. cenocepacia* H111, as it is generally believed?

The answer to this question seems to depend on the type of biofilm examined. If we only consider the "classical" biofilm model, i.e. a submerged biofilm at the solid-liquid interphase, our results oppose the general textbook knowledge. Although the intracellular c-di-GMP concentrations were found to be increased in both the *rpfR* and the *rpfF$_{Bc}$* mutant, the amount of biofilm formed by these mutants was lowered relative to the wild type. In literature, there are a few examples, where decreased c-di-GMP levels stimulate biofilm formation. For example, mutation of the gene encoding the GGEEF protein PA3343 in *P. aeruginosa*, which is an active diguanylate cyclase, has been reported to result in hyperbiofilm formation whereas over-expression of PA2879 and PA3343, which both lead to increased intracellular levels of c-di-GMP, had no effect on biofilm formation (Kulasekara *et al.*, 2006). However, when the formation of a pellicle is considered to be an indicator of biofilm formation at the

liquid-air interphase then the classical textbook knowledge that a high c-di-GMP level correlates with sessility does apply for *B. cenocepacia* H111.

Things get more complicated when looking at strains in which RpfR or its variants were expressed *in trans*. Here, the measured c-di-GMP levels do not always match the expectations. The complementation of the *rpfR* mutant with a constitutively expressed RpfR protein led on the one hand to the restoration of a wild-type phenotype in all three biofilm models, bacterial motility and proteolytic activity (Figure 2, Figure 5). On the other hand, instead of a decreased intracellular c-di-GMP level, this strain exhibited increased levels relative to the mutant (even though deviation between replicates was high). Addition of extra BDSF reduced the intracellular c-di-GMP level, but not to level of the wild-type. This could be due to an increased *rpfR* copy number, as this gene was expressed from a *lac* promoter. Under this condition it is likely that the natural pool of BDSF gets depleted (Figure 4). Likewise, when the GGDEF domain of RpfR was mutated, the intracellular c-di-GMP level remained at the level of the mutant and did not drop to the wild-type level, as would have been expected when an EAL domain is expressed.

When pBBR5-rpfR$_{AAL}$ or pBBR-PA5295 is present in the *B. cenocepacia* H111 wild type, the intracellular c-di-GMP concentration is dramatically increased or decreased, respectively. The colony morphology, however, is smooth in both strains and pellicles are not as robust as in the *rpfR* mutant, but remain fragile or are completely absent. Together with the fact that a submerged biofilm is affected by expression of pBBR-rpfR$_{AAL}$ in the wild-type background, this suggests a dual role of RpfR. Firstly, RpfR alters gene expression in response to the BDSF signalling molecule by altering c-di-GMP levels. This in turn effects the expression of *cepI* and thus also the AHL regulon. Secondly, it seems that the function of RpfR in addition to c-di-GMP also depends on another mechanism, likely specific protein-protein interactions.

A recent study describes a novel concept in which, likewise to the two putative functions of RpfR, the enzymatic activity of a PDE/DGC enzyme is linked to protein-protein interaction (Lindenberg *et al.*, 2013). The subject of this study was YciR of *E. coli*, to which *rpfR* shows 54% identity in amino acid sequence and the same protein domain architecture. In *E. coli*, the expression of the key biofilm regulator CsgD regulator is, in addition to other factors, regulated by two multi-gene modules. Module I consists of YegE and YhjH, module II of YdaM and YciR. YciR comprises two activities: on the one hand, it binds and degrades c-di-GMP generated by module I and on the other hand, YciR inhibits the diguanylate cyclase YdaM by direct interaction. This second activity is relieved when YciR is acting as active phosphodiesterase under high c-di-GMP conditions and thus allows YdaM to generate

c-di-GMP and to activate another transcription factor which then drives csgD transcription (Lindenberg *et al.*, 2013). Hence, YciR not only links modules I and II, but also functions as a trigger enzyme, i.e. an enzyme that besides its enzymatic activity also contributes to the regulation of gene expression (Commichau & Stülke, 2008). With this definition, RpfR may also represent a trigger enzyme. Whether the concept of YciR is applicable for RpfR remains to be determined and current work in our lab aims to elucidate this.

4.2 QS in *B. cenocepacia* H111: an entangled web of BDSF, AHL and c-di-GMP signalling

In Schmid *et al.* (2012), we examined in detail the overlap of the BDSF and the AHL regulons. We employed RNA-Seq and shutgun proteomics to assess the BDSF regulon of *B. cenocepacia* H111 and compared it to the previously published AHL regulon. The rationale for this study was the observation that phenotypes of the *rpfR* or the $rpfF_{Bc}$ mutant, such as reduced motility, impaired biofilm formation, lowered proteolytic activity and attenuated virulence are also found in mutants of the AHL-QS system. The two regulons show considerable overlap (Figure 10) and very importantly, BDSF and its cognate receptor RpfR regulate the expression of *cepI* through alteration of c-di-GMP levels. Nevertheless, by using a variety of promoter fusions and the single or double mutant of either QS molecule synthase, we were able to show that in case of *bapA* or the *bclACB* operon, both signalling molecules are required for maximal gene expression. Thus, the two QS systems don't act hierarchically, but rather in parallel. Two recent studies performed in other laboratories confirmed these results. In *B. cenocepacia* J2315, the expression of the genes encoding for the AHL synthases CciI and CepI were found to be reduced in an $rpfF_{Bc}$ mutant (Udine *et al.*, 2013). The paper of Deng *et al.* (Deng *et al.*, 2013) also confirmed the influence of BDSF on AHL levels in *B. cenocepacia* H111 and showed that this is caused by the negative regulatory effect of c-di-GMP on *cepI* transcription. In addition, they found a cumulative effect of the two QS systems on biofilm formation, protease activity and swarming motility.

To gain a deeper insight into the involvement of c-di-GMP in gene regulation, we profiled gene expression in *B. cenocepacia* wild-type derivatives with altered intracellular c-di-GMP levels. By using a ≥ 3 fold change cutoff, we found 15 genes, for which an increase in c-di-GMP levels led to increased expression and 98 genes, where an increase in c-di-GMP levels led to reduced expression. As expected, *cepI* was among the most highly affected

genes. Consequently, we found a substantial overlap with the AHL regulon published earlier (Inhülsen *et al.*, 2012). Given that the BDSF QS system alters the c-di-GMP levels, we expected to find an overlap between the c-di-GMP transcriptome and the BDSF regulon. Indeed, 43 genes were found to be regulated by both systems. There are several reasons for the fact that not all of the BDSF-regulated genes were found among the c-di-GMP regulated genes and *vice versa*. Firstly, in strain J2315, there is an additional, subordinate BDSF receptor, the histidine kinase BCAM0227, described, whose signal transduction is independent of c-di-GMP (McCarthy *et al.*, 2010). Secondly, as mentioned above, RpfR is likely to act also *via* an unknown c-di-GMP-independent mechanism and therefore not all genes that are regulated by the BDSF-RfpR system are also c-di-GMP regulated. Thirdly, in both transcriptome analyses, stringent criteria were applied to filter for differentially expressed genes; hence some jointly regulated genes may not have passed all the filter criteria. Fourthly, genome analysis using the Conserved Domain Database (Marchler-Bauer *et al.*, 2011) revealed that *B. cenocepacia* H111 encodes 23 proteins that are putatively involved in c-di-GMP metabolism as listed in Table 5. Artificial modification of c-di-GMP levels may therefore also lead to differential expression of genes whose expression is, under natural conditions, stimulated by other c-di-GMP proteins.

Among the exclusively c-di-GMP regulated genes, the whole phenylacetate degradation pathway was found to be down-regulated in the strain with high c-di-GMP levels. The phenylacetate catabolic pathway is a central route through which different aromatic compounds, such as styrene, phenylethylamine or phenylalanine, are aerobically degraded and funnelled into the TCA cycle (Teufel *et al.*, 2010) and it has been subjected to several studies in *B. cenocepacia* strains K56-2 and J2315 (Hamlin *et al.*, 2009; Hunt *et al.*, 2004; Imolorhe & Cardona, 2011; Law *et al.*, 2008; Sass *et al.*, 2011; Yoder-Himes *et al.*, 2009; Yudistira *et al.*, 2011). In *B. cenocepacia*, the phenylacetate degradation pathway is encoded by genes arranged in three clusters (Figure 11), which are repressed by glucose and succinate, whereas they are induced in synthetic CF sputum medium (Hamlin *et al.*, 2009; Law *et al.*, 2008; Yoder-Himes *et al.*, 2009). Interestingly, this pathway has been associated with pathogenicity of *B. cenocepacia*. For example, the gene *paaE* was found to contribute to persistence of *B. cenocepacia* in the lungs of a rat and a mutant of *paaE* performed worse in a competition assay with the wild-type strain (Hunt *et al.*, 2004). Likewise, inactivation of *paaE* or *paaA* resulted in attenuation in a *C. elegans* infection model (Law *et al.*, 2008). In addition, the three gene clusters encoding the phenylacetate degradation pathway were found to be up-regulated in a mutant spontaneously resistant to the antibiotic meropenem (Sass *et al.*, 2011).

Table 5. All EAL and/or GGDEF domain containing proteins of *B. cenocepacia* H111. Domain analysis was performed with CCE identifiers using the Conserved Domain Database (Marchler-Bauer *et al.*, 2011), searching for entries cd01948, cd01949 and cl00290. Additionally, 7 HDc superfamiliy proteins (cl17215) were identified; however, none of them contained the GYP sig nature motif characteristic for the HD-GYP domain**.**

ID[a]	Description	ids J2315 orthologs	EAL	GGDEF
CCE46345	Diguanylate cyclase/phosphodiesterase domain 1 (GGDEF)	BCAM2822		X
CCE46768	Rtn protein	BCAM0158	X	
CCE47054	EAL domain protein	BCAM1670		X
CCE47190	Diguanylate cyclase/phosphodiesterase domain 2 (EAL)	BCAM1554		X
CCE47660	Sensory box/GGDEF family protein	BCAM2426	X	
CCE47702	diguanylate cyclase (GGDEF domain) with PAS/PAC sensor	BCAM1161		X
CCE47703	PAS/PAC domain protein	BCAM1160	X	X
CCE47761	GGDEF domain protein	BCAL1635		X
CCE48054	EAL domain protein	BCAL0652	X	
CCE48088	EAL domain protein	BCAL0621	X	X
CCE48447	RpfR	BCAM0580	X	X
CCE48941	Diguanylate cyclase/phosphodiesterase domain 1 (GGDEF)	BCAM0748		X
CCE49211	diguanylate cyclase (GGDEF domain) with PAS/PAC sensor	BCAM2836		X
CCE49426	GGDEF domain protein	BCAL1975		X
CCE49595	EAL domain protein	BCAL2749	X	
CCE49999	EAL domain protein	BCAL3188	X	
CCE50580	Sensory box/GGDEF family protein	BCAL2449	X	X
CCE51514	EAL domain protein	BCAL1100	X	
CCE51548	EAL domain protein	BCAL1069	X	X
CCE51594	GGDEF domain protein	BCAL1020; BCAL2852		X
CCE52221	GGDEF domain protein	BCAS0398		X
CCE52847	putative transmembrane protein	BCAL0430		X
CCE52990	Inner membrane protein YfiN	BCAM2256		X

[a]: Nomenclature according to GenBank file CAFQ01000000.

Four genes encoding components of the type III secretion system (T3SS) were down-regulated under high c-di-GMP condition. T3SSs, which have evolved to deliver proteins from the bacterial cytoplasm into the host cell cytosol, are found in plant as well as in animal pathogens (Galán, 1999). In *B. cenocepacia* the T3SS is poorly characterised. A mutant in *bscN*, which encodes a component of the T3SS of *B. cenocepacia* J2315, was attenuated in a mouse infection model, with significantly lower bacterial recovery from the lungs and spleens and less pronounced histopathological changes of lung tissue when compared to the wild type (Tomich *et al.*, 2003). Furthermore, a *bscN* mutant was found to show reduced killing of *C. elegans* and to be less competitive than the K56-2 wild type in the *D. melanogaster* infection model (Castonguay-Vanier *et al.*, 2010; Uehlinger *et al.*, 2009). Interestingly, while some

genes of the T3SS are BDSF-regulated in *B. cenocepacia* J2315, they are not in strain H111 (McCarthy *et al.*, 2010; Schmid *et al.*, 2012).

Unsurprisingly, alteration of the c-di-GMP levels strongly impacts the physiology of *B. cenocepacia* H111. Very high c-di-GMP concentrations correlate with abolished proteolytic activity, severe defects in swarming and swimming motility, reduced siderophore production and diminished biofilm formation. On the other extreme, a low level of intracellular c-di-GMP led to enhanced bacterial motility and the inability to form pellicles (Figure 13). Some of the mentioned phenotypes and several single genes identified as c-di-GMP-regulated contribute to the pathogenicity of *B. cenocepacia*. Testing of strains with altered intracellular c-di-GMP concentrations revealed that the c-di-GMP levels of *B. cenocepacia* H111 negatively correlate with pathogenicity in *C. elegans* and *G. mellonella*. The strain H111*(rpfR$_{AAL}$)* was completely attenuated in both infection models, whereas H111(PA5295) was as virulent as the wild type.

Chromosome 3 of *B. cenocepacia* has recently been reclassified as a virulence plasmid, termed pC3. Mutants with deleted pC3 are non-pathogenic in the *G. mellonella* infection model (Agnoli *et al.*, 2012). Hence, there must be some virulence factors and/or regulators encoded by pC3. Interestingly, we identified only *zmpA* and the *aidA* gene as known pathogenicity factors among the c-di-GMP regulated genes on pC3. None of them, however, contribute to pathogenicity in this infection model (Uehlinger *et al.*, 2009). Likewise, many other of the known genes contributing to pathogenicity of *B. cenocepacia* against *G. mellonella* are not among the c-di-GMP regulated genes, for example: *orbA* (BCAL1700), *pvdA* (BCAL1699), *hldA* (BCAL2945) (Uehlinger *et al.*, 2009); flagellum genes *fliC* (BCAL0114) and *fliJ* (BCAL0521), LPS core polysaccharide genes *hldA* (BCAL2945) and *waaC* (BCAL3112) (Loutet & Valvano, 2010); BCAM0227 (McCarthy *et al.*, 2010); BCAM0224 (Mil-Homens & Fialho, 2011). However, one of the c-di-GMP regulated genes, *bceF*, was recently shown to contribute to the virulence of *B. cepacia* IST408 in the *G. mellonella* infection model (Ferreira *et al.*, 2013). It is tempting to speculate that down-regulation of the cepacian cluster I, to which *bceF* belongs, is responsible for the decreased pathogenicity of the strain when the intracellular c-di-GMP levels are high.

The present study contributes to the understanding of the QS network in *B. cenocepacia*. By using biochemical and molecular biological approaches, we identified a novel component of the BDSF QS circuitry, the BDSF receptor protein RpfR, which affects the intracellular

second messenger c-di-GMP. We found that the BDSF- and AHL-dependent QS systems can act either together or independently, as the depicted in the model shown in Figure 18. This multicomponent network may offer enhanced flexibility in the diverse environments in which *B. cenocepacia* can exist.

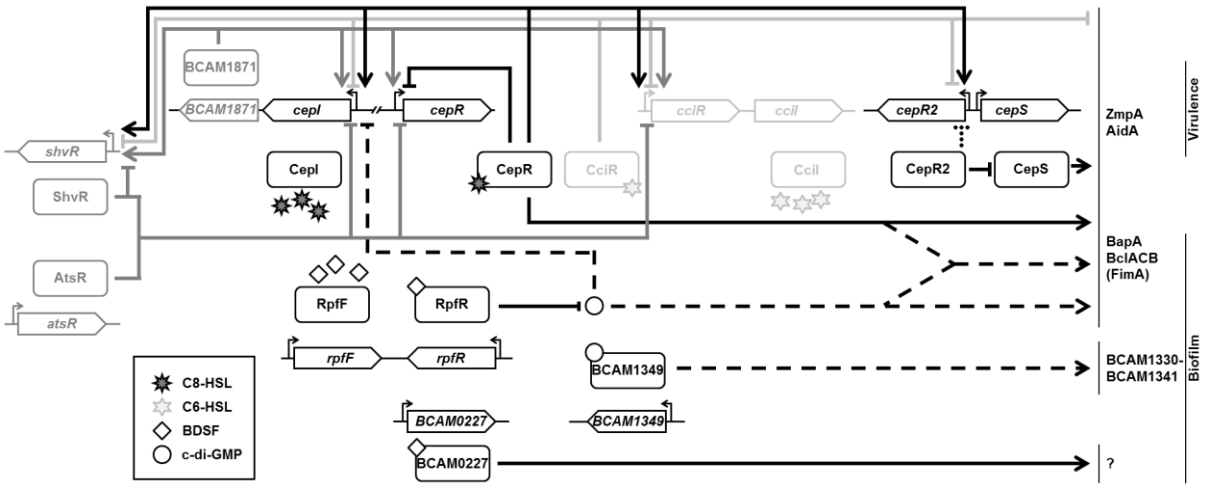

Figure 18. Model of the QS network in *B. cenocepacia*. Depicted in black is the "core" QS circuitry of *B. cenocepacia* that is present in all strains and that consists of the CepIR and the RpfFR systems, as well as the downstream regulators CepR2, CepS, BCAM1349 and BCAM0227. The regulators ShvR, AtsR and BCAM1871, which fine-tune AHL-dependent QS, are shown in dark gray. The second AHL-dependent QS system, CciIR, which is only present in strains of the ET-12 lineage, is shown in light gray. The dashed lines indicate regulation of target genes through the second messenger c-di-GMP. The dotted line indicates contradictory experimental data regarding the autoregulation of *cepR2*. Figure from (Suppiger *et al.*, 2013).

5. Future prospects

The findings in the present thesis revealed that the BDSF and the AHL QS systems of *B. cenocepacia* H111 are interlinked *via* the second messenger c-di-GMP. However the precise mechanism by which c-di-GMP influences gene expression is not known yet. Thus, further efforts should focus on the identification and characterization of the downstream regulatory elements of the c-di-GMP signalling cascade. So far, there is only one transcriptional regulator of *B. cenocepacia* described that directly links c-di-GMP sensing to gene regulation. At high intracellular c-di-GMP levels BCAM1349, a protein with an N-terminal cNMP domain and a C-terminal DNA binding domain, binds the dinucleotide. As consequence, expression of the cellulose synthase-encoding *bsc* operon and the exopolysaccharide-encoding gene cluster BCAM1330-41 was suggested to be stimulated (Fazli *et al.*, 2011, 2012). This regulator, however, does not seem to be affected by the AHL- or the BDSF-dependent QS system.

Identification of this downstream regulatory element(s) may also answer other questions connected to c-di-GMP. It is still puzzling how specificity of the c-di-GMP signal is achieved, given the fact that usually multiple GGDEF, EAL and/or HD-GYP proteins are present within one organism. *B. cenocepacia* H111 encodes for 22 such proteins in addition to RpfR (see Table 5). Possible explanations involve spatial or temporal resolution of the DGC/PDE proteins or different affinities to c-di-GMP. In this context, the work of Lindenberg and coworkers (Lindenberg *et al.*, 2013) is particularly interesting, as it revealed a new mechanism of c-di-GMP signalling that is based on modular protein-protein interactions. It would be very interesting to see if RpfR is involved in such a modular regulatory mechanism.

6. Material and Methods

6.1 Bacterial strains, plasmids, oligonucleotides and growth conditions

Bacterial strains, plasmids and oligonucleotides used are listed in Table 6 and Table 7.
Unless otherwise stated, strains were grown aerobically at 37°C in LB Lennox broth (Difco) with shaking at 225 rpm or on LB Lennox plates (1.5% agar). For counterselection of *E. coli* in triparental conjugations, PIA (Pseudomonas isolation agar, Difco) plates were used. Complementation assays were performed with 10 µM BDSF (Sigma) and/or 200 nM C8-HSL (Sigma). Antibiotics were used at the concentrations indicated in
Table 8. Growth was monitored spectrophotometrically by measurement of optical density at 600 nm (Ultrospec Pro 2100, GE Healthcare, Switzerland).

Table 6. Bacterial strains and plasmids used in this study.

Strain	Phenotype and/or characteristic	Reference
Burkholderia cenocepacia		
bapA	*bapA*::Km mutant of H111	(Inhülsen *et al.*, 2012)
BCAM0227	Insertional mutant with Bcam0227 interrupted by pEX18Gm	(Deng *et al.*, 2012)
cepR	*cepR*::Km mutant of H111, Km^R	(Huber *et al.*, 2001)
H111	wild type, CF isolate from Germany, genomovar III	(Gotschlich *et al.*, 2001; Römling *et al.*, 1994)
H111(pBBR5)	H111 with pBBR1MCS-5	This study
H111(PA5295)	H111 harboring pBBR5-PA5295	This study
H111(*rpfR*$_{AAL}$)	H111 harboring pBBR5-rpfR$_{AAL}$	This study
H111(*rpfR*$_{GGAAF}$)	H111 harboring pBBR-rpfR$_{GGAAF}$	This study
H111-AidA	*aidA*::Km mutant of H111	(Huber *et al.*, 2004)
iclR	H111 insertional mutant with iclR (I35_5431, BCAL1082 (pseudogene)) interrupted by pEX18Gm	This study
m17	H111 transposon mutant with *rpfR* (formerly *yciR*) interrupted	(Huber *et al.*, 2002)
m17(*rpfR*)	m17 complemented with pBBR-rpfR	This study
rhlA	H111 insertional mutant with rhlA (BCAM2340) interrupted by pEX18Gm	This study
rhlB	H111 insertional mutant with rhlB (BCAM2338) interrupted by pEX18Gm	This study
rpfF$_{Bc}$	*rpfF*$_{Bc}$::pSHAFT2 mutant of H111, Cm^R	(Schmid *et al.*, 2012)
rpfR	H111 insertional mutant with rpfR interrupted by pEX18Gm	(Deng *et al.*, 2012)
rpfR(*rpfR*)	Mutant rpfR harboring the expression construct pBBR-rpfR	(Deng *et al.*, 2012)
rpfR(*rpfR*$_{AAL}$)	Mutant rpfR harboring the expression construct pBBR-rpfR$_{AAL}$	(Deng *et al.*, 2012)
rpfR(*rpfR*$_{GGAAF}$)	Mutant rpfR harboring the expression construct pBBR-rpfR$_{GGAAF}$	(Deng *et al.*, 2012)

Table 6 (continued)

ΔcepI	Δ*cepI* mutant of H111, markerless	(Schmid *et al.*, 2012)
ΔcepI *rpfF*$_{Bc}$	Δ*cepI* and rpfF$_{Bc}$::pSHAFT double mutant, Cm^{R}	(Schmid *et al.*, 2012)
Escherichia coli		
CC118 λpir	Δ*(ara, leu)7697 araD139* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB(RfR) argE(am) recA1* λpir^{+}	(Herrero *et al.*, 1990)
DH5α	F^{-} Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) *recA1 endA gyrA96 thi-1 hsdR17 supE44 relAl deoR(U169)*	(Hanahan, 1983)
HB101	F^{-} *supE44 hsdS20* (r$_{B}^{-}$ m$_{B}^{-}$) *recA13 ara-14 proA2 lacY1 galK2, rpsL20 xyl-5 mtl-1 recA thi pro leu hsdR*$^{-}$ *M*$^{+}$ Sm$^{r;}$	(Boyer & Roulland-Dussoix, 1969)
M15[pREP4]	NaI^{S}, Str^{S},Rif^{S}, Thi^{-}, Lac^{-}, Ara^{+}, Gal^{+}, Mtl^{-}, F^{-}, RecA^{+}, Uvr^{+}, Lon^{+}, pREP4	Quiagen
MM294	F^{-} *endA1 hsdR17 supE44*(AS*) rfbD1 spoT1 thi-1*	(Meselson & Yuan, 1968)
OP50	uracil auxotroph, food source for *C. elegans*	(Brenner, 1974)
S 17-1	thi *recA* pro hsdR^{-} hsdM^{+} RP4-2-Tc::Mu-Km::Tn7	(Simon *et al.*, 1983)
XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔ*M15* Tn*10* Tet^{r}	Stratagene
other organisms		
Pseudomonas aeruginosa PAO1	wild-type *P. aeruginosa*	(Holloway, 1955)
Pseudomonas putida F117	IsoF, mutation in *ppuI*, (AHL-negativ)	(Steidle *et al.*, 2001)
Caenorhabditis elegans N2 Bristol	wild-type *C. elegans*	*Caenohabditis* Genetic Center, USA
Galleria mellonella	wild type	Brumann Fischerei & Sportartikel AG, Switzerland
Plasmids		
pAS-C8	pBBR1MCS-5 carrying P$_{cepI}$::*gfp*(ASV) P$_{lac}$::*cepR*	(Riedel *et al.*, 2001)
pBBR1MCS	broad host-range cloning vector	(Kovach *et al.*, 1994)
pBBR1MCS-5	broad host-range cloning vector	(Kovach *et al.*, 1995)
pBBR5-PA5295	pBBR1MCS-5 expressing PA5295 from *P. aeruginosa*	Prof. Urs Jenal, University of Basel, unpublished
pBBR5-rpfR$_{AAL}$	pBBR5-rpfR harboring an E443A amino acid substitution	This study
pBBRcepI	pBBR1MCS-5 containing the *cepI* gene of *B. cenocepacia* H111	(Schmid *et al.*, 2012)
pBBR-rpfR	pBBR1MCS containing *rpfR* of H111	(Huber *et al.*, 2002)
pBBR-rpfR$_{AAL}$	pBBR-rpfR harboring an E443A amino acid substitution	(Deng *et al.*, 2012)
pBBR-rpfR$_{GGAAF}$	pBBR-rpfR harboring a D318A and E319A amino acid substitution	(Deng *et al.*, 2012)
pEX-0227	pEX18Gm containing an internal fragment of *BCAM0227*	(Deng *et al.*, 2012)
pEX18Gm	pUC18 MCS, oriT^{+}, *sacB*$^{+}$; gene replacement vector	(Hoang *et al.*, 1998)
pEX-iclR	pEX18Gm containing an internal fragment of *iclR*	This study
pEX-rhlA	pEX18Gm containing an internal fragment of *rhlA*	This study
pEX-rhlB	pEX18Gm containing an internal fragment of *rhlB*	This study
pEX-rpfR	pEX18Gm containing an internal fragment of *rpfR*	(Deng *et al.*, 2012)
pGEM-T easy	TA cloning vector	Promega
pP_{aidA}-*lacZ*	pSU11 containing the putative *aidA* promoter region	(Inhülsen *et al.*, 2012)
pP_{bapA}-*lacZ*	pSU11Tp containing the putative *bapA* promoter region	(Inhülsen *et al.*, 2012)
pP_{bclA}-*lacZ*	pSU11 containing the *bclA* promoter region	(Inhülsen *et al.*, 2012)
pP_{cepI}-*lacZ*	pSU11 containing the *cepI* promoter region	(Schmid *et al.*, 2012)
pP_{rhlA}-*lacZ*	pSU11 containing the *rhlA* promoter region	This study

Table 6 (continued)

pQE-RpfR	pQE-32 containing *rpfR* of H111	This study
pQE-RpfR$_{AAL}$	pQE-RpfR harboring an E443A amino acid substituti	This study
pQE-RpfR$_{GGAAF}$	pQE-RpfR harboring a D318A and E319A amino acid substitution	This study
pRK2013	RK2 derivative, *mob*$^+$ *tra*$^+$ *ori* ColE1	(Figurski & Helinski, 1979)
pRK600	RK2-*mob*$^+$ RK2-*tra*$^+$, *ori* ColE1	(Kessler *et al.*, 1992)
pRN3	source of dhfr cassette	(O'Grady *et al.*, 2009)
pSHAFT2	Broad-host-range suicide plasmid, mobilisable for conjugation	S. Shastri / M.S. Thomas, manuscript in preparation
pSHAFT-rpfF$_{BC}$	pSHAFT2 containing an internal fragment of *rpfF*$_{Bc}$	(Schmid *et al.*, 2012)
pSU11	promoter probe vector	(O'Grady *et al.*, 2009)
pSU11Tp	pSU11 derivative harboring dhfr cassette from pRN3	(Schmid *et al.*, 2012)
pUT-miniTn5	transposon delivery vector	(Herrero *et al.*, 1990; de Lorenzo *et al.*, 1990)

Table 7. Oligonucleotides used in this study.

Primer	Sequence (5'-3')[a]
for cloning of insertional mutants	
rpfR_F	ggatccGAAGTGAATTCGCTCGTCGT
rpfR_R	aagcttGAGATCGAGGAACAGGATGC
rpfR_check	GTCGGCAGGAAGGTCAATAC
$rpfF_{Bc}$_F	ggatccTTCAACCAGCAACTCGTCAC
$rpfF_{Bc}$_R	aagcttCCGCAGTCTTCGTACCACTC
$rpfF_{Bc}$_check	AAGCTTAACCACATCTCACGAGGACA
BCAM0227_F	ggatccGTGATCGACAGCGAGAACCT
BCAM0227_R	aagcttCTTCGACAGGTCGAGGATGT
BCAM0227_check	ATTCCCGAAACCGACTGG
bapR_F	ggatccAATCGTCACCCGACGAGAT
bapR_R	aagcttTTCGGCCAGCTCGATCAT
bapR_check	aagcttTCGAGTTTCGGAATTAGAGAGA
rhlA_F	ggatccGCGATGGTCGACTACGTGA
rhlA_R	aagcttGCTTCGCCACAGAAGAAATC
rhlA_check	GCCAGACCGTCCAGTATCTC
rhlB_F	ggatccACTGGCCGTCGAATCATCT
rhlB_R	aagcttGTGACGACCTGCACGATG
rhlB_check	CGCTGATGACGCTGATCG
pSHAFT_check	AAGGTGACCGCGTATTATTA
pEXcheckF	GTGCTGCAAGGCGATTAAGT
for heterologous expression and mutagenesis of RpR	
pQE-rpfR_F	ggatccATATGGATGACGAAAACGATAGCGCG
pQE-rpfR_R	aagcttTCAGGCGATCAGCCTGAGCT
AAL_F	GGCGACGTGCACGGCGTCGCGGCGCTGATCCGCCAGTCG
AAL_R	CGACTGGCGGATCAGCGCCGCGACGCCGTGCACGTCGCC
GGAAF_F	GCTCGCGCGGCTCGGCGGCGCCGCATTCCTCGTGCTGTTCGAAC
GGAAF_R	GTTCGAACAGCACGAGGAATGCGGCGCCGCCGAGCCGCGCGAGC
for construction of pSU11Trp	
dhfR_F	gcatgcGGTCTGACGCTCAGTGGAACG
dhfR_R	gcatgcGCTTAGGCCACACGTTCAAG
for construction of pP_{rhlA}-lacZ	
PrhlA_F	ctcgagGGTCGAAGCAGATCGTGTT
PrhlA_R	aagcttAATCAACAAAAAATCGGGGC
for identification of transposon insertion site	
ARB2	GGCCACGCGTCGACTAGTAC
ARB6	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
arb-sp-int	GACCTTGCCATCATGACTGTGCTG
arb-sp-ext	AACGCGTATTCAGGCTGACC
arb-seq	ATGAATGTTCCGTTGCGCTG

[a]: restriction sites used for cloning are in lower case

Table 8. Concentration of antibiotica used.

Antibiotic	*E. coli* strains	*B. cenocepacia* H111 strains
Ampicillin	100 µg/ml	not used (intrinsically resistant)
Chloramphenicol	20 µg/ml	60-80 µg/ml
Gentamicin	10 µg/ml	10 µg/ml
Kanamycin	25 µg/ml	50-100 µg/ml
Trimethoprim	25 µg/ml	50-100 µg/ml

6.2 Molecular methods

6.2.1 PCR reaction purification, plasmid preparation and gel extraction

PCR reaction purification, plasmid preparation and gel extraction was performed with kits obtained from QIAgen, according to the manufacturer's protocol.

6.2.2 General cloning protocols

For cloning purposes, the following enzymes were used according to the manufacturer's protocol:

Table 9. Enzymes used for cloning.

Purpose	Enzyme	Supplier	Remarks
PCR	GoTaq DNA Polymerase	Promega	5% DMSO
PCR	Phusion High-fidelity DNA Polymerase	NEB*	HF buffer, 3% DMSO, if proofreading quality needed
Restriction digest	various restriction endonucleases	NEB*	
Dephosphorylation	TSAP	Promega	
Ligation	T4 DNA ligase	Promega	

* NEB: New England Biolabs

6.2.3 Genomic DNA extraction

For total DNA extraction, the protocol described by Better *et al.* (1983) was modified as follows: 200 µl of an overnight culture of *B. cenocepacia* was harvested by centrifugation and the pellet was resuspended in 200 µl TE-buffer (50 mM Tris, 20 mM EDTA, pH 8), followed by the addition of 250 µl sarkosyl solution (2% sodium *N*-lauroylsarcosinate in TE-buffer) and 50 µl pronase solution (5 mg/ml pronase in TE-buffer, predigested 30 min at 30°C). The mixture was incubated at 37°C until it cleared up. The lysate was sheared by vortexing for 2

min and extracted with 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1) by vortexing and centrifugation. Approximately 300 µl of the aqueous phase was transferred into a fresh tube and the DNA was precipitated with 25 µl of 5 M NaCl solution and 350 µl isopropanol. The DNA was harvested with a small plastic loop, washed in ice cold ethanol and resuspended in 500 µl double distilled H_2O (ddH_2O).

6.2.4 Sequencing

DNA Sequencing was performed according to Sanger's dideoxynucleotide chain termination method (Sanger & Nicklen, 1977) using the in-house sequencing service on the ABI 3730 DNA sequencer at the Institute of Plant Physiology (University of Zurich, Switzerland) or sending the samples to Microsynth AG (Balgach, Switzerland). For in-house sequencing, the samples were prepared by the follwing PCR reaction:

ABI Big Dye®	0.8 µl
5x Sequencing buffer	1.5 µl
Primer (5 pmol/µl)	0.25 µl
DNA (100 – 180 ng)	1.0 µl
ddH_2O	6.45 µl

Thermocycler program:

Initial denaturation:	94 °C	2 min	
Denaturation	96 °C	10 s	} 60x
Annealing	according to primer	5 s	
Elongation	60 °C	3 min	

Sequences obtained were analysed with CLC main workbench (CLCbio).

6.2.5 Competent cells, transformation

Competent *E. coli* were prepared and transformed according to Chung *et al.*(Chung *et al.*, 1989).

6.2.6 Conjugative plasmid transfer

Plasmid conjugations from *E. coli* to *B. cenocepacia* were performed with helper strains *E. coli* MM294 (pRK2013) or *E. coli* HB101 (pRK600). Briefly, 2-4 ml overnight culture of the donor strain, the helper strain, and the *Burkholderia* recipient strain were harvested and washed twice in LB medium to get a final volume of 500 µl. 100 µl of the donor and the helper strain were mixed and 200 µl of the recipient strain was added. Then the mixture was transferred onto LB plates in 50 µl spots. After incubation at 37°C for at least 6 h (or

overnight) the bacteria were resuspended in 0.9% sodium chloride, plated on PIA plates containing the appropriate antibiotics and incubated at 37°C.

6.2.7 Construction of *B. cenocepacia* H111 mutants

All mutants were generated by single homologue recombination of a suicide vector into the target gene. Thereto, an internal fragment of approx. 300 to 500 bp length was amplified by PCR with primers containing restriction sites (Table 7). Those PCR fragments were ligated into pGEM-T easy (Promega) according to the manufacturer's instructions and transformed into *E. coli* DH5α. From clones containing the correct insert (as verified by PCR), plasmid DNA was prepared and digested with the respective restriction enzymes. The fragments were purified and ligated into the respective sites of the suicide vectors pEX18Gm or pSHAFT2 and transformed into *E. coli* DH5α and *E. coli* CC118 λpir, respectively. The plasmid was transferred to the respective *B. cenocepacia* H111 strain (wild type or mutant) by triparental conjugation and transconjugants were selected on PIA medium with the appropriate antibiotics.

6.2.8 Site directed mutagenesis of RpfR for heterologous expression and for complementation studies

The site-directed mutagenesis of RpfR on plasmid (pQE-32 or pBBR1MCS) was generated on basis of the QuikChange site directed mutagenesis system (Agilent) as described in Deng *et al.* (2012) with primer pairs AAL_F/AAL_R and GGAAF_F/GGAAF_R.

6.2.9 Construct for RpfR protein purification

The *rpfR* gene was amplified using the primer pair pQE-rpfR-fw and pQE-rpfR-rev and cloned into pGEM-T (Promega). The construct was then digested with *Bam*HI and *Hind*III and the released fragment cloned into pQE-32, digested with the same enzymes. The plasmid termed pQE-RpfR was transformed into *E. coli* M15[pREP4] and confirmed by sequencing. Plasmids pQE-$RpfR_{AAL}$ and pQE-$RpfR_{GGAAF}$ were generated by site-directed mutagenesis of pQE-RpfR likewise to pBBR-rpfR as described in 6.2.8 with primer pairs AAL_F/AAL_R and GGAAF_F/GGAAF_R. The plasmids were then extracted from *E. coli* XL1blue and transformed into *E. coli* M15[pREP4].

6.2.10 Transposon mutant screening

6.2.10.1 Construction of the transposon mutant library

For construction of a pooled transposon library, a conjugative plasmid transfer was carried out as described in 6.2.6, with *E. coli* S17 pUT-miniTn5 as donor strain and the BDSF and AHL double negative strain *B. cenocepacia* H111 $rpfF_{Bc}$ ΔcepI as recipient strain. The bacterial mix was plated on PIA plates containing chloramphenicol (60 µg/ml) and Kanamycin (100 µg/ml) and after overnight incubation, the colonies were counted (46100 colonies) and washed from the plates with LB Lennox broth. For stock conservation, 0.5 volumes of 50% glycerol were added to the pooled library, and the suspension was aliquoted and kept at -80°C.

6.2.10.2 Screening for clones that do not longer respond to exogenous BDSF

The reporter plasmid pP_{bapA}-*lacZ* was delivered to the pooled transposon library by triparental plasmid transfer. For recovering of the library pool, 1.5 ml of the cryo-conserved library pool was given into 8 ml LB and incubated for 30 min (to avoid excessive growth) with gentle shaking (50 rpm). Then, 2 ml of an overnight culture of the donor strain, *E. coli* DH5α pP_{bapA}-*lacZ* and the helper strain, as well as 4 ml of the library pool were mixed and the plasmid transfer was carried out as described in 6.2.6. After overnight incubation, different volumes of the bacterial mix were plated on PIA plates containing trimethoprim (100 µg/ml), X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 100 µg/ml) and BDSF (10 µM) to determine the efficiency of the plasmid transfer, while the remainder was kept in 0.9 % sodium chloride at room temperate. The next day, the remainder was plated on identical plates to aim well separated single colonies. This yielded in approx. 86000 colonies and after two days of incubation, colonies that did not respond to the BDSF added to the plate, i.e. the colonies that were pale blue instead of intense blue, were streaked on fresh PIA plates containing in addition to trimethoprim, X-gal and BDSF also chloramphenicol (60 µg/ml) and Kanamycin (50 µg/ml).

6.2.10.3 Identification of the transposon insertion site

The transposon insertion sites of the selected clones were identified by arbitrary PCR. The conditions used are listed below. As template total genomic DNA from the selected clones, extracted as described in 6.2.3, was used. After the first and the second round PCR, the PCR

reaction was purified with the PCR purification kit (Qiagen). Sequencing was performed in-house with the usual protocol (see 6.2.4) and primer arb-seq.

1st round PCR mix:

DNA	2	µl
dNTPs (10 mM each)	2	µl
5x buffer (goTaq, flexi)	10	µl
DMSO	4	µl
$MgCl_2$ (25 mM)	6	µl
Primer 1: ARB6 (50 µM)	4	µl
Primer 2: arb-sp-int (5 µM)	4	µl
GoTaq DNA polymerase	0.5	µl
ddH_2O	17.5	µl

1st round PCR thermocycler program:

95°C	5	min	
95°C	0.5	min	6 x
30°C	0.5	min	
72°C	1	min	
95°C	0.5	min	30x
45°C	0.5	min	
72°C	1	min	
72°C	5	min	
12°C	forever		

2nd round PCR mix:

purified 1st round PCR product	5	µl
dNTPs (10 mM each)	2	µl
5x buffer (goTaq, flexi)	10	µl
DMSO	4	µl
$MgCl_2$ (25 mM)	6	µl
Primer 1: ARB2 (50 µM)	4	µl
Primer 2: arb-sp-ext (5 µM)	4	µl
GoTaq DNA polymerase	0.5	µl
ddH_2O	14.5	µl

2nd round PCR thermocycler program:

95°C	5	min	
95°C	0.5	min	30x
50°C	0.5	min	
72°C	1	min	
72°C	5	min	
12°C	forever		

6.2.11 Transcriptome analysis

The transcriptome analysis of *B. cenocepacia* H111 wild type harbouring either pBBBR-PA5295 (low c-di-GMP level), pBBR1MCS5 (normal c-di-GMP level) and pBBR-rpfR$_{AAL}$ (high c-di-GMP level) was performed in two completely independent replicates, whereas the transcriptome analysis of *B. cenocepacia* H111 wild type (without plasmid) and the *iclR* mutant were performed only once.

6.2.11.1 Growth conditions, RNA extraction and DNAse treatment

A pre-culture of the strains tested were grown to an OD_{600} of approx. 1.5 and then used to inoculate 100 ml of LB Lennox medium at an OD_{600} 0.05. After growth to late exponential growth phase (OD_{600} 2.0), 13.5 ml of the culture were rapidly transferred to cold tubes containing 1.5 ml of "stop solution" (10% phenol buffered with 10 mM Tris-HCl, pH 8, in ethanol), centrifuged 5 min at 5000 rpm at 4°C and the pellet frozen in liquid nitrogen. Total RNA was extracted using a hot acid phenol protocol. Briefly, the pellet was resuspended in 1.5 ml ice cold buffer A (20 mM sodium acetate pH 5.5, 1 mM EDTA) and added to a mixture of 160 µl 10% SDS, 2 ml buffer A and 3.5 ml acid phenol. The suspension was vigorously mixed for 30 s and incubated for 7 min at 65°C with an additional vortexing step of 1 min in between. The aqueous phase was separated and re-extracted twice; first with 3 ml phenol/chloroform/isoamylalcohol and then with 2.5 ml chloroform. The total RNA was then precipitated at -80°C overnight with 1/10 volume of 3 M sodium acetate pH 5.5 and 2 volumes of 100% ethanol.

Quality control for the RNA was performed by using an RNA Nano Chips (Agilent 2100 Bioanalyzer; RIN >8).

To remove the 5S rRNA that would interfere with the subsequent mRNA enrichment procedure, 50 µg of total RNA in a volume of 100 µl was run over the columns of the RNAeasy MiniKit (Qiagen), according to the manufacturer's protocol, and eluted twice with each 30 µl of elution buffer.

To (further) deplete the DNA, 30 µg of the RNA were treated with RQ1 RNAse-Free DNAse I (Promega) and SUPERaseIn RNAse Inhibitor (Ambion) for 30 min at 37°C and purified with the RNAeasy MiniKit (Qiagen). The successful depletion of DNA was confirmed by PCR using primers rhlA_F and rhlA_R (target: *rhlA*) with 35 cycles.

6.2.11.2 Depletion of mRNA, cDNA synthesis and library generation

The depletion of mRNA was performed by using the MICROB*Express* Kit (Ambion). For each strain, 2 to 3 reactions with each 5 µg of RNA were used and greatest care was taken to strictly follow the protocol of the manufacturer. If subsequent bioanalyzer results suggest an incomplete mRNA removal, this step was repeated with pooled samples.
For the synthesis of cDNA and library generation, the Ovation Prokaryotic RNA-Seq System (Nugen) and the Ovation Ultralow Library Systems (Nugen) were used. Again, all measures were taken to ensure the strict adherence to the two protocols.

6.2.11.3 RNA-Sequencing and data analysis

The libraries sequenced in one sequencing lane were pooled to each 10 nM. The sequencing was performed at the FGCZ, University of Zurich, on an Illumina HiSeq 2000, with 100 bp single-end reads and provided to us in fastq format. The sequencing reads were mapped to the *B. cenocepacia* H111 genome using CLC Genomics. The number of reads per gene was normalized to the total number of reads in each sample and to the length of the gene to yield an RPKM value (Mortazavi *et al.*, 2008). For calculation of the fold change of a particular gene expressed in the wild type and the *iclR* mutant, the ratio of the RPKM values of the gene was calculated. Orthologue mapping and functional classification was performed as described previously (Schmid *et al.*, 2012). Read mapping, RPKM value and fold change calculation, orthologue mapping and functional classification was done by Gabriella Pessi.
To obtain a list with differentially expressed genes as response to changes in c-di-GMP levels, the unique read counts of strains H111(PA5295), H111(pBBR5) and H111($rpfR_{AAL}$) were analysed with the R-package DESeq2 (Anders & Huber, 2010). Only genes with an adjusted p-value <0.05 (corresponds to an FDR (False Discovery Rate) of <5%) were considered.
To generate the Venn diagram with overlap of this data with the *cepR* regulon and the BDSF stimulon, the gene list (Table 3) was compared to the raw data of the *cepR* microarray as deposited in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under the ID number E-MTAB-509 and to the transcriptome data of Schmid *et al.* 2012.

6.3 Protein biochemical methods

6.3.1 Heterologous expression and purification of RpfR and its variants

For expression of the 6xHis-fusion protein, an overnight culture of the E. coli M15[pREP4] pQE-RpfR was diluted 1:100 into LB Lennox supplemented with ampicillin and kanamycin, and grown at 37°C (with shaking at 225 rpm) to an OD600 of 1.0. Protein expression was induced by adding IPTG at a final concentration of 10 µM and incubating at 23°C with shaking at 225 rpm for 18 h. Then, 450 ml of bacterial culture was harvested by centrifugation (15 min, 6000 rpm, 4°C) and the cell pellet was resuspended in 12 ml lysis buffer (50 mM NaH_2PO_4 pH 8.0, 500 mM NaCl, 5 mM Imidazole, 10% Glycerol) containing one Complete Mini Protease inhibitor tablet, EDTA free (Roche). The cells were passed twice through a cell disruptor (Constant Cell Disruption System, Constant Systems) at 2 kbar and the lysate was centrifuged (60 min, 16000 rpm, 4°C) to obtain the soluble protein in the supernatant.
Purification of the protein was obtained by using a batch purification protocol. Briefly, 1.1 ml of resin (Profinity IMAC Ni-charged, Biorad) was prepared as recommended by the manufacturer, following the batch-mode purification protocol, with the exception that instead of vacuum, gentle centrifugation was applied. Next, 1 ml of the 50% slurry was added to cleared lysate and mixed with mild shaking for 60 min at 4°C. The resin was collected by centrifugation at 1000 g and washed with washing buffer (50 mM NaH_2PO_4 pH 8.0, 1 M NaCl, 30 mM Imidazole) three times with a total of 60 CV. Elution was performed 5 times with each 0.5 ml elution buffer (50 mM NaH_2PO_4 pH 8.0, 1 M NaCl, 250 mM Imidazole). Following the purification, the buffer was exchanged by dialysis over night at 4°C against dialysis buffer (200 mM Tris-Cl pH 7.8, 500 mM NaCl, 8 mM $MgCl_2$) in dialysis tubes (Dialysis tubing cellulose membrane, D9277, 10 mm, Sigma-Aldrich) prepared according to the manufacturer's protocol.
The purified and dialysed protein was analysed on a 12%-SDS-PAGE gel.

6.3.2 DGC / PDE assay

To assess the enzymatic activity of RpfR, a DGC / PDE assay was performed as previously described (Spangler *et al.*, 2010). Briefly, 2 µM of purified RpfR or its variants were resuspended in 50 mM Tris-Cl pH 7.8, 500 mM NaCl, 2 mM $MgCl_2$. BDSF was added to a final concentration of 10 µM where indicated. The reaction was started by adding the substrate (GTP (Sigma) or c-di-GMP (Biolog)) at a final concentration of 100 µM and by immediately incubating the reaction at 37°C. After 180 min of incubation, the protein was

denatured by heating to 99°C for 5 min, followed by centrifugation at 20000 g for 15 min. Supernatant was then analyzed on an Äkta FPLC system (1 ml ResourceQ column (GE Healthcare), linear gradient from 0.5%-100% of 1 M $(NH_4)HCO_3$ pH 8). GTP, c-di-GMP and pGpG (Biolog) were used as standards in a concentration of 100 µM.

6.3.3 Western blot

Bacterial cultures were grown overnight in LB medium and a volume that corresponds to 13 µl of an OD_{600} of 4.0 was run on a 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham HybondTM-P, GE Healthcare) by using the Trans-blot Turbo Blotting System (BioRad) and Twobin transfer buffer (Towbin *et al.*, 1979). Membranes were incubated with anti-AidA antibodies (in a dilution of 1:2000) as primary antibody and HRP-linked anti-rabbit IgG as secondary antibody. Detection was performed with Immuno Star HRP (BioRad) according to the instruction of the manufacturer.

6.4 Phenotypic analyses

6.4.1 Analyis of gene expression by measuring β-galactosidase activity

The β-galactosidase activity was measured as described in Schmid *et al.* (2012).

6.4.2 Extraction and Quantification of c-di-GMP

An overnight culture of the strains to be analyzed was diluted to an OD_{600} of 0.05 in LB Lennox and grown to late exponential growth phase. OD_{600} was recorded and 5 ml of culture was harvested by centrifugation at 4°C. Nucleotide extraction was performed as described by Spangler *et al.* (Spangler *et al.*, 2010) with slight modifications: cXMP was omitted and evaporation to dryness was performed using a vacuum concentrator.
For quantification by LC-MS/MS, the dried extracts were sent to Annette Garbe, Prof. Dr. Volkhard Kaever laboratory, Medizinische Hochschule Hannover, Germany.

6.4.3 Extraction and detection of rhamnolipids

Bacterial cultures were grown overnight in nutrient broth (0.5% peptone, 0.3% beef extract) and harvested. The separated and sterile filtered supernatant was acidified with 10 M HCl to reach approx. pH 2 and extracted twice with each 0.5 volumes of chloroform:methanol 2:1. The lower organic phases were collected and subsequently split into 2 ml Eppendorf tubes

and evaporated to dryness in a vacuum concentrator at 45°C. The rhamnolipid extracts were redissolved in methanol to reach 200 fold concentration.

The detection of rhamnolipids was performed by TLC. To this end, 5 µl (*P. aeruginosa*, as control) or 20 µl (*B. cenocepacia*) of the extract was spotted on a TLC 60 Kieselgel plate (Merck, Darmstadt, Germany) and developed by using a mixture of 80% chloroform, 18% methanol and 2% acetic acid as mobile phase. To visualize the carbohydrate moiety of the rhamnolipids, either an anthrone reagent or a molish reagent was prepared and sprayed on the plates. For the preparation of the anthrone reagent, 200 mg of anthrone was dissolved in 1.65 ml of ethanol. Next, 4.05 ml ethanol, 0.4 ml of water and 0.65 ml of concentrated H_2SO_4 were mixed. 1.05 ml of this mix was then added to the previously prepared anthrone solution to yield the anthrone reagent. Stained carbohydrate spots appear in a blue-green colour on the TLC plate. The molish reagent was prepared by adding 5 to 8 drops of concentrated H_2SO_4 to a 10% solution of α-naphthol in ethanol. Carbohydrates react with this reagent to give a purple colour.

6.4.4 Analysis of protease activity

6.4.4.1 Qualitative determination of proteolytic activity on skim milk plates

Bacterial overnight culture (5 µl) was spotted on a skim milk plate (LB Lennox, 2 % skim milk, 1.0 % agar) and incubated for 24 hours at 37°C before photographic documentation. The assay was repeated at least three times.

6.4.4.2 Quantitative determination of proteolytic activity with azocasein

Quantification of protease activity was carried out as described in Schmid *et al.* (2012).

6.4.5 Biofilm quantification

Biofilm assays were performed as described by Schmid *et al.* (2012).

6.4.6 Analysis of bacterial motility

6.4.6.1 Swarming motility

Swarming motility was determined on semi-solid NB (nutrient broth) plates (0.5% peptone, 0.3% beef extract, 0.5% agar). 5 µl of bacterial overnight culture were inoculated at the centre of the plates and incubated for 24 h in humid conditions at 30°C. Then the plates were either documented photographically or the diameter of the swarming zone was recorded in two approx. orthogonal axes and the average of those values was used as swarming zone diameter. Data show representative plates and values based on at least three independent experiments with each at least three technical replicates, respectively.

6.4.6.2 Swimming motility

Swimming motility was determined on semi-liquid NB plates (0.5% peptone, 0.3% beef extract, 0.3% agar). The plates were inoculated by pricking a toothpick that was previously dipped into bacterial overnight culture into the centre of the plate. After 24 h incubation at 30°C, the diameter of the swimming zone was determined and/or the plates were photographed. The assay was performed at least three times independently with each at least three technical replicates.

6.4.7 Analysis of AHL production

6.4.7.1 Qualitative AHL detection by cross streak

For the qualitative detection of AHL molecules, the test strain and the sensor strain *P. putida* F117 pAS-C8 were streaked on a LB agar plates in a 90° angle without contact. The plates were incubated for 24 h at 30°C and analysed for GFP (green fluorescent protein) expression. Documentation was performed with a Leica M165FC Zeiss binocular microscope equipped with a Leica DFC310 FX Zeiss camera.

6.4.7.2 Quantitative AHL detection in liquid culture

AHL quantity in liquid cultures was determined as described in Schmid *et al.* (2012).

6.4.8 Pellicle formation

For assessing pellicle formation, an overnight culture of the test strain was diluted 1:100 in NYG broth (0.5% peptone, 0.3% yeast extract, 2% (w/v) glycerol) and incubated in a closed vessel without shaking at room temperature for approx. 5 days. The pellicle formed was then categorized by visual assessment into three classes: no pellicle (no cells floating on the liquid medium), thin pellicle (a thin opaque layer formed at the liquid-air interface, very fragile, detaches easily when moving the vessel, usually wrinkly appearance), robust pellicle (a thick, robust layer formed at the liquid-air interface, does not detach even when slightly tilting the vessel, usually smooth appearance with single folds).

6.4.9 Colony morphology on NYG plates

Colony morphology was determined on NYG plates (0.5% peptone, 0.3% yeast extract, 2% (w/v) glycerol, 1.5% agar). Bacterial overnight culture (2 - 5µl) was spotted on the plates and incubated for 3 days at 37°C followed by 2 days at room temperature.

6.4.10 Siderophore detection

The siderophore production of bacteria was analysed employing the chrome azurol S (CAS) assay (Schwyn & Neilands, 1987) with the following modification: the agar component was prepared with LB-pipes agar (7.65 g PIPES (Sigma), 2.5 g Bacto tryptone, 1.25 g Bacto yeast extract, 1 g NaCl in 225 ml H2O) instead of MM9 salts and casamino acids. When iron is removed from the original CAS-Fe(III) complex due to siderophore production, blue-to-orange halos surround bacterial colonies. Five µl of bacterial overnight culture was spotted on the plates and incubated for 24-48 h at 37°C.

6.5 Pathogenicity assays

Pathogenicity assays with *Caenorhabditis elegans* and with *Galleria mellonella* were performed as described by Agnoli and coworkers (Agnoli *et al.*, 2012). The *G. mellonella* assay in Figure 13 was performed by Roman Freitag.

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8. Appendix

8.1 List of figures and tables

Figures

Tables

8.2 Book chapter

Schmid, N., Pessi, G., Aguilar, C., & Eberl, L. (2014). Cell-to-cell Communication and Biofilm Formation of Members of the Genus Burkholderia: A Story of Multilingually Talented Bacteria. In T. Coenye & E. Mahenthiralingam (Eds.), Burkholderia: From Genomes to Function. Caister Academic Press. In press.

Cell-to-cell communication and biofilm formation of members of the genus *Burkholderia*: a story of multilingually talented bacteria

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Abstract

Members of the genus *Burkholderia* appear to be particularly loquacious. Evidence has accumulated over the past few years that these bacteria are capable of utilizing at least three different chemical languages: *N*-acyl homoserine lactones, *cis*-2-unsaturated fatty acids and quinolones. Here we wish to summarize the current knowledge of the underlying molecular architectures of these communication systems and show that they are involved in the control of some highly conserved functions, including the production of extracellular proteolytic enzymes, motility, antifungal activity, expression of virulence factors and biofilm formation. Particular attention is paid to the role of these communication systems in the formation of surface-associated consortia and the genes that are required for the sessile lifestyle of this group of bacteria. The new emerging role of the intracellular secondary messenger cyclic diguanylate (c-di-GMP) in biofilm formation and especially as a downstream regulatory element of the fatty acid signaling cascade is discussed. Finally, given that these communication systems are required for both biofilm formation and pathogenicity, they are suggested to represent highly valuable targets for the development of novel antibacterial drugs.

Members of the genus *Burkholderia* speak multiple languages

N-acyl homoserine lactone (AHL)-based QS systems in Burkholderia sp.

AHL-based QS systems typically rely on two proteins: an AHL synthase and an AHL receptor protein. At low cell-densities the expression of the AHL synthase gene, coding for a protein of the LuxI family, occurs at a basal level such that the signal molecules slowly accumulate in the culture medium. However, upon reaching a critical threshold concentration, the AHL molecule binds to its cognate receptor, a protein of the LuxR family of transcriptional regulators. This complex in turn binds to specific sites in the promoter regions of target genes, activating or repressing their transcription (Williams, 2007). First indications that a *Burkholderia* species produces AHLs were obtained in a study that showed that the culture supernatant of a *B. cepacia* strain was able to activate different AHL biosensors (McKenney et al., 1995). A genetic screen for mutants with altered siderophore production in *Burkholderia cenocepacia* K56-2, eventually lead to the identification of the CepI/R system (Lewenza et al., 1999). The AHL synthase, CepI, was shown to direct the synthesis of *N*-octanoyl-homoserine lactone (C8-HSL) and minor amounts of *N*-hexanoylhomoserine lactone (C6-HSL) (Figure 1). Subsequent studies revealed that the CepR/AHL complex binds as a dimer to an imperfect palindromic sequence that often partially overlaps the -35 regions of target promoters, thereby initiating transcription of downstream genes (Chambers et al., 2006; Wei et al., 2011). This recognition sequence, referred to as *cep* box, is also present in the promoter region of *cepI*. Thus, once the QS system is triggered a positive feedback loop is established that leads to a rapid increase in AHL levels and consequently in target gene expression. In addition, the CepR/AHL complex also negatively controls its own expression (Lewenza and Sokol, 2001).

The CepI/R system appears to be present in all 17 *Burkholderia cepacia* complex (Bcc) species, although direct experimental evidence is missing for a few of them, namely *B. arboris, B. diffusa, B. latens, B. metallica,* and *B. seminalis* (Gotschlich et al., 2001; Venturi et al., 2004; Wopperer et al., 2006). CepI/R orthologs have also been identified in all members of the pseudomallei group, consisting of *B. pseudomallei*, *B. mallei*, *B. thailandensis* and *B. oklahomensis,* as well as in in some species of the recently defined plant-beneficial-environmental *Burkholderia* group (PBE), although it remains to be seen whether they are true orthologs or belong to independent evolutionary lineages (Suárez-Moreno et al., 2010). Recent work has also provided evidence that many members of the PBE group harbour another highly conserved LuxIR pair, designated BraI/R, that relies on the AHL signal 3-oxo-C14-HSL (Suárez-Moreno et al., 2008). Finally, many *Burkholderia* strains have been demonstrated to harbour more than one AHL-dependent QS system and produce multiple AHL signal molecules (Riedel and Eberl, 2007). It is beyond the scope of this article to summarize all these systems and we will rather focus on *B. cenocepacia*, which is the best-investigated model organism.

In addition to the CepI/R system, *B. cenocepacia* strains belonging to the ET12 lineage carry the CciI/R QS system on the *B. cenocepacia* island (cci), a 31.7-kb low GC content pathogenicity island encoding 35 putative ORFs (Baldwin et al., 2004). This QS system differs from the CepI/R system in that *cciI* and *cciR* form a transcriptional unit, whereas *cepI* and *cepR* are divergently transcribed (Malott et al., 2005). Furthermore, the main product of CciI is C6-HSL, while C8-HSL, the main product of CepI, is only produced in minor amounts (Malott et al., 2005). The CepI/R QS system and the CciI/R QS system do not act independently: CepR is required for transcription of the *cciIR* operon, whereas

CciR negatively controls the expression of CepI, but not of CepR (Malott et al., 2005). Results from a transcriptomic analysis suggested a reciprocal regulation of target genes by the two QS systems, which ensures the fine-tuned expression of target genes (O'Grady et al., 2009).

Over the past few years it has become evident that the *B. cenocepacia* QS circuitry is far more complex than initially anticipated, as various higher-level regulatory elements as well as downstream regulators were identified. An additional regulatory element influencing the activity of the CepI/R QS system is ShvR, a LysR type transcriptional regulator that was initially shown to be responsible for shiny colony variants of *B. cenocepacia* K56-2 (Bernier et al., 2008). In a recent study it has been shown that ShvR controls the expression of more than 1000 genes (O'Grady et al., 2011), among them was *ccil*/*R* and *cepI*/*R,* although the effect on the latter system was only weak. Consequently, ShvR affects AHL kinetics: maximal AHL production peaked earlier in a *shvR* mutant compared to wild type (O'Grady et al., 2011). However, the regulons of ShvR and CepR/CciR only partially overlap and phenotypic analyses of mutants defective in *shvR*, *cepR* or *cciR* suggest that ShvR not only influences expression of CepR- and CciR-regulated genes, but also of a set of genes which is not under direct QS control (O'Grady et al., 2011).

In addition to ShvR, another global regulator, AtsR, was shown to modulate QS signalling. This response regulator was first identified as a repressor of biofilm formation and was shown to control expression of a type VI secretion system (Aubert et al., 2008). A recent study demonstrated that deletion of *atsR* led to increased expression of *cepI/R* and *ccil/R*, which in turn led to premature and increased AHL production. This finding suggests that AtsR plays a role in controlling the timing and fine-tuning of AHL-dependent gene expression. However, AtsR is also able to repress QS-regulated genes independently of AHL production, indicating that AtsR may operate *via* a novel yet uncharacterized mechanism (Aubert et al., 2012).

In contrast to ShvR and AtsR, which down-regulate both QS systems, a recent study identified a regulator that promotes transcription of *cepI/R* and *ccil/R* in *B. cenocepacia* K56-2 (O'Grady et al., 2012). This regulator, BCAM1871, is co-transcribed with *cepI* and acts as an enhancer of AHL production (positive feedback). Mutants in BCAM1871 showed reduced transcription levels of *ccilR* and of *shvR*. However, and similar to AtsR, some phenotypes were affected independently of AHL levels or CepI/R function, suggesting that BCAM1871 may operate through an AHL-independent mechanism (O'Grady et al., 2012).

As many other Proteobacteria, *B. cenocepacia* harbors an orphan or solo *luxR* gene, i.e. a *luxR* gene not associated with a cognate *luxI* AHL synthase gene (for a review see (Subramoni and Venturi, 2009).This LuxR solo, designated CepR2, is a 237 amino-acid ortholog of CepR and contains all the conserved residues of LuxR transcriptional regulators (Malott et al., 2009). In *B. cenocepacia* K56-2, CepR2 is negatively regulated by CciR and by itself, and it does not affect expression of the CepI/R or the CciI/R system. CepR2 was shown to be both a negative and a positive regulator of gene expression and does not require AHL to exert its function (Malott et al., 2009). Interestingly, the set of genes regulated by CepR2 partially overlaps with those of the CepI/R and CciI/R regulons, including the genes encoding the metalloproteases ZmpA and ZmpB and the nematocidal factor AidA.

Fatty acid signalling in Burkholderia sp.

In 2008, Boon et al. discovered that some *Burkholderia* strains produce a long chain fatty acid signal molecule, the structure of which was identified as *cis*-2-dodecenoic acid, referred to as BDSF (*Burkholderia* diffusible signal factor). BDSF is a structural and functional analogue of *cis*-11-methyl-2-dodecenoic, the diffusible signal factor (DSF) of *Xanthomonas campestris* pv. *campestris* (Xcc), which is a well characterized QS signal in this organism. Analyses of structural derivatives of DSF revealed that the key features of this type of QS signal molecules in Xcc are the double bond between C1 and C2, as well as its *cis*-conformation (Wang et al., 2004), both features are present in BDSF. Genetic analysis revealed that in *B. cenocepacia* J2315, the gene responsible for BDSF synthesis is BCAM0581. This gene codes for an enoyl-CoA hydratase that shares 37% identity at the amino acid level with RpfF, the main DSF synthase of Xcc. *In trans* expression of BCAM0581 in a DSF-deficient Xcc strain can rescue the defects of the mutant, including biofilm formation and extracellular polysaccharide production (Boon et al., 2008). BCAM0581 has been recently renamed to $rpfF_{Bc}$ and shown to encode a bifunctional enzyme that catalyses the dehydration of 3-hydroxydodecanoyl-ACP to *cis*-2-dodecenoyl-ACP and cleaves the thioester bond to give BDSF (Bi et al., 2012). Besides BDSF, some *Burkholderia* strains also produce additional DSF-family signals like DSF or *cis,cis*-11-methyldodeca-2,5-dienoic acid (DSF with an additional double bond in *cis*-configuration between C5 and C6) (Deng et al., 2010). Examination of nine species from the Bcc (*B. lata*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, *B. anthina* and *B. pyrrocinia*) showed that all produced BDSF, with four of them (*B. multivorans*, *B. stabilis*, *B. anthina*, and *B. pyrrocinia*) additionally producing *cis,cis*-11-methyldodeca-2,5-dienoic acid and *B. multivorans* producing DSF (Deng et al., 2010). Interestingly, the observed diversity of DSF-family signal molecules in Bcc species is not related to variations found in the $RpfF_{Bc}$ primary structure, but is likely due to the availability of different precursors in the different Bcc species (Deng et al., 2010). Blast analyses revealed orthologues of $RpfF_{Bc}$ from *B. cenocepacia* J2315 in all members of the *Pseudomallei* group, in two pathogenic *Burkholderia* species (*B. glumae* and *B. rhizoxinica*) and in members of the PBE cluster (*B. graminis*, *B. phymatum*, *B. phytofirmans*, *B. terrae* and *B. xenovorans*) (Winsor et al., 2008; Deng et al., 2012).

Although Xcc is using a similar signal molecule as *B. cenocepacia*, the signal receptor as well as the signaling cascade is completely different. The DSF receptor of Xcc, RpfC, is a hybrid sensor kinase that phosphorylates its cognate response regulator RpfG. In addition to a receiver domain RpfG contains a HD-GYP domain, which was shown to be responsible for the c-di-GMP phosphodiesterase activity of the protein thereby affecting the cellular c-di-GMP level (Ryan and Dow, 2010). However, neither *rpfC* nor *rpfG* orthologs could be identified in any of the sequenced Bcc genomes. However, the fact that the gene next to $rpfF_{Bc}$ (BCAM0580, named *rpfR*) is required for biofilm formation (Huber et al., 2002), a phenotype also known to be affected by BDSF (Ryan et al., 2009; Deng et al., 2010), prompted a more detailed study that revealed that BCAM0580 encodes a novel fatty acid signal receptor (Deng et al., 2012). RpfR is a protein that comprises a PAS, a GGDEF and an EAL domain. The latter two are known to be responsible for c-di-GMP synthesis and degradation, respectively. A *B. cenocepacia* H111 *rpfR* mutant phenocopies a BDSF-deficient mutant, including an increased intracellular c-di-GMP level, reduced motility, impaired biofilm formation, and lowered proteolytic activity. Biochemical characterization of the purified RpfR protein demonstrated that BDSF binds to the

PAS domain with high affinity and stimulates the phosphodiesterase activity of the protein through induction of allosteric conformational changes. RpfR represents the first example of a c-di-GMP metabolic enzyme that is directly activated by cell-cell communication signals via protein-ligand interaction. A bioinformatic analysis revealed that the *rpfF*$_{Bc}$*/rpfR* gene cluster is not only highly conserved in 11 *Burkholderia* species (*B. cenocepacia*, *B. cepacia*, *B. multivorans*, *B. dolosa*, *B. ambifaria*, *B. vietnamiensis*, *B. ubonensis*, *B. phymatum*, *B. xenovorans*, *B. graminis* and *B. phytofirmans*), but also in members of other genera, including *Achromobacter*, *Yersinia*, *Serratia*, *Enterobacter*, *Pantoea*, and *Cronobacter* (Deng et al., 2012).

Quinolone-based QS in Burkholderia sp.

In addition to AHL- and BDSF-based QS systems, a third potential QS system, which relies on quinolone signal molecules, was identified in some *Burkholderia* strains. The archetypal quinolone-based QS system is the one of *Pseudomonas aeruginosa,* which utilizes 2-heptyl-3-hydroxy-4(1*H*)-quinolone (also known as the Pseudomonas quinolone signal, PQS) to control expression of many virulence genes. PQS is also considered an integral component of the AHL-dependent QS network of this organism (Diggle et al., 2006). The biosynthesis of PQS relies on the *pqsABCD* gene products, which direct the synthesis of 2-heptyl-4-quinolone (HHQ), the precursor of PQS synthesis. HHQ is thought to be an extracellular messenger that is released from, and taken up by, bacterial cells, where it is converted into PQS. Using LC-MS/MS analysis it was shown that several *B. pseudomallei* strains, *B. cenocepacia, B. thailandensis* and *B. ambifaria* produce, in addition to HHQ, other 4-hydroxy-2-alkylquinoline derivatives and also 4-hydroxy-3-methyl-2-alkylquinolines but not PQS (Diggle et al., 2006; Vial et al., 2008). Mutants of *B. ambifaria* defective in quinolone signalling were shown to have an increased production of AHLs and, consequently, slightly up-regulated expression of AHL-dependent phenotypes (Vial et al., 2008). In *B. pseudomallei* mutation of the *pqsA* homologue not only resulted in the loss of HHQ production but also in altered colony morphology and increased elastase production (Diggle et al., 2006). Additional work will be required to elucidate the molecular mechanisms underlying the quinolone signalling cascade interaction with the AHL- and BDSF-dependent QS circuitries.

QS-regulated phenotypes and regulons

The impact of QS on the physiology of *Burkholderia* species has been assessed by phenotypic characterization as well as by global profiling approaches (transcriptomes or proteomes) using defined QS mutants. The majority of these studies focused on pathogenic species, including Bcc species, the pseudomallei group and the rice pathogen *B. glumae*. In table 1 we show QS-regulated phenotypes of different *Burkholderia* species. Some of the listed functions, including biofilm formation, proteolytic activity, motility, antifungal activity and pathogenicity are highly conserved, suggesting that they represent ancestral QS-regulated phenotypic traits.

QS-regulated functions were also mapped by functional genomics approaches. A transcriptomic analysis of CepR-regulated genes in *B. cenocepacia* H111 identified 57 genes that were more than threefold up- or down-regulated in a *cepR* mutant relative to the wild type strain (Inhülsen et al., 2012). The same custom *B. cenocepacia* oligonucleotide microarray was also used to identify the overlap between the CepI/R and CciI/R systems in *B. cenocepacia* K56-2 (O'Grady et al., 2009). When

compared with the wild type, 860 genes were found to be more than twofold up- or down-regulated in a K56-2 *cepR* mutant, 595 genes in a *cciR* mutant and 489 genes in a *cepR cciR* double mutant (O'Grady et al., 2009). Among those genes, 196 genes were regulated by both CepR and CciR, with the vast majority (167) being positively regulated by CepR and negatively regulated by CciR. With only very few exceptions all 57 CepR-regulated genes identified in strain H111 (Inhülsen et al., 2012) were also among the CepR-regulated genes of strain K56-2. Both studies showed that *aidA*, which encodes a factor required for nematode pathogenicity, was among the most stringently CepR-activated genes. Interestingly CciR had a negative effect on *aidA* transcription in strain K56-2. The dual regulation of *aidA* in K56-2 provides an explanation for the observation that the *cepI* mutants of H111 and K56-2 but not the K56-2 *cciI* mutant are attenuated in virulence in the *C. elegans* infection model (Uehlinger et al., 2009). Other AHL-regulated genes that were identified in both studies include several proteases, an operon encoding three lectins, a type I pilus, a non-ribosomal peptide synthetase gene cluster, and genes required for swimming motility (Inhülsen et al., 2012; O'Grady et al., 2009).

Global proteomic analyses have been used to study the QS regulon of *B. cenocepacia* H111. An early study by Riedel et al. (2003) employed two-dimensional gel-electrophoresis (2-DE) to compare the proteomes of the H111 wild type and its isogenic *cepI* mutant grown in presence or absence of AHLs. Fifty five of the 985 detected protein spots were found to be differentially regulated. Due to limited genome sequence data at that time, only of the 19 QS-regulated proteins could be identified. In good agreement with the more recent transcriptomic data (Inhülsen et al., 2012; O'Grady et al., 2009), the proteins included the ZmpB metalloprotease, the FimA protein, the BclA lectin and AidA. A more recent study, employing a gel-free proteomics methodology, identified 22 proteins that were down-regulated in a *cepI* as well as in a *cepR* mutant of *B. cenocepacia* H111 (Inhülsen et al., 2012). This analysis not only provided confirmatory results but also showed that many of the QS-regulated proteins were not among the QS-regulated genes identified by the microarray analyses, suggesting that expression of many of the identified proteins may be controlled by QS at the posttranscriptional level.
2-DE was also employed to identify AHL-regulated proteins in *B. glumae* BGR1 (Goo et al., 2010). Among 79 differentially expressed proteins identified, 59 were extracellular proteins, such as flagellin, lipases, proteases, proteins involved in anti-oxidation and membrane proteins. The high proportion of extracellular proteins among the QS-regulated proteins was elegantly explained by the finding that expression of the type II secretion system, which is required for secretion of many extracellular proteins, is controlled by QS (Goo et al., 2010). Likewise, 2-DE was used to map the proteins controlled by one (the *bpsI/R* system) of the three AHL-dependent QS systems in *B. pseudomallei* (Wongtrakoongate et al., 2012). Forty five of the 65 identified genes were found to be also regulated by the stationary sigma factor RpoS, most likely because RpoS is involved in the regulation of *bpsI* expression.

In a recent study the BDSF stimulon of *B. cenocepacia* H111 was mapped by RNA-Seq and shotgun proteomics (Schmid et al., 2012). These investigations provided evidence that many of the BDSF-regulated genes or proteins are also controlled by the CepI/R system, suggesting that the two regulons partially overlap. This is in accordance with previous studies that have shown that AHLs and

BDSF control similar phenotypic traits, including biofilm formation, proteolytic activity and pathogenicity (Deng et al., 2009, 2012, Figure 2).The detailed analysis of two mutually regulated operons, one encoding three lectins and the other one encoding the large surface protein BapA and its type I secretion machinery, revealed that both AHLs and BDSF are required for full expression, suggesting that the two signalling systems operate in parallel (Figure 3). In accordance with this, it was demonstrated that both AHLs and BDSF are required for biofilm formation and protease production. Even though AHL production is reduced by 50% in the *rpfF*$_{Bc}$ mutant, the two QS systems were found to regulate the tested phenotypes and genes independently, suggesting that they are not hierarchically arranged.

Molecular mechanisms and factors important for biofilm formation of Burkholderia sp.

As pointed out above, one of the phenotypes regulated by both the AHL- and BDSF-dependent QS system (no data on the role of quinolone signalling are available) is the formation of surface-associated communities, referred to as biofilms. In nature bacteria are thought to exist predominantly as biofilms on both biotic and abiotic surfaces. Biofilms are also of eminent clinical importance, as sessile cells are up to 1000-fold more resistant to antibiotics than their planktonic counterparts. In the following we will discuss factors that have been reported to play a role in biofilm formation of *Burkholderia* sp.

Pili – surface appendages

The adhesion to surfaces is often mediated by specialized proteinaceous surface appendages such as fimbriae or pili or in some bacteria also flagella (Watnick and Kolter, 2000). *Burkholderia* sp. expresses flagella and various types of pili (Goldstein et al., 1995). Using transmission electron microscopy (TEM), five types of pili were identified in Bcc strains: mesh (Msh), filamentous (Fil), spine (Spn), spike (Spk), and cable (Cbl) pili. The expression of these different pili types has been linked to the ecological niches from which the respective strains have been isolated from. Msh pili have been shown to be produced by many clinical and environmental strains, whereas Cbl pili were only expressed by epidemic CF isolates. Fil pili were found to be associated with non-epidemic CF isolates, Spn pili with non-CF clinical isolates, and Spk pili were exclusively produced by environmental strains. The cable pilus Cbl has been demonstrated to be important for adhesion of Bcc strains to epithelial cells and is therefore thought to be of particular clinical relevance (Sajjan et al., 2002). It is noteworthy that only a few Bcc strains harbour the gene coding for the Cbl pilus and not all stains possessing the gene produce pili (Sajjan et al., 2002).

Many *Burkholderia* strains produce a homolog of the *E. coli* FimA protein, the major subunit of the type I pilus. In *E. coli* this pilus is critical for initial attachment to biotic and abiotic surfaces (Pratt and Kolter, 1998). A FimA homolog in *B. cenocepacia* H111 was identified among the CepR-regulated genes (Riedel et al., 2003). *fimA* (BCAL1677) is the first gene of an operon that also contains genes encoding for a chaperone-usher secretion apparatus (BCAL1678-1681) (Holden et al., 2009; Inhülsen et al., 2012). Regulatory elements like homologs of FimB and FimE, which control phase variation in

E. coli, or a tip fibrillum gene are missing in *B. cenocepacia* H111. In spite of the fact that type I fimbriae are crucially important in some organisms for adhesion to surfaces, a *fimA* mutant of *B. cenocepacia* H111 was neither defective in biofilm biomass nor in biofilm architecture (Inhülsen et al., 2012). As the analysis of the H111 genome indicated the presence of at least two additional chaperon-usher-type pili, two Flp-type pili, a type IV pilus, it is conceivable that these additional surface appendages may mask the biofilm defects of the *fimA* mutant. In *B. pseudomallei*, the type IV pilin-encoding gene *pilA* was shown to be essential for microcolony development, and thus optimum association with eukaryotic cells, but is not required for direct adherence to cultured human cells (Boddey et al., 2006).

Genes required for biofilm maturation

Using a simple microtiter dish based assay Huber et al. (2002) screened a random transposon insertion library of *B. cenocepacia* H111. Given the relatively long incubation time (48 hours), mutants defective in the late steps of biofilm formation were enriched in this screen. Of 5000 transposon insertion mutants screened, 13 exhibited defects in biofilm formation on a polystyrene surface without being impaired in growth. However, all the mutants were defected in the development of the typical three-dimensional biofilm structure of *B. cenocepacia* H111. The 13 mutants were shown to carry the transposon in genes encoding surface proteins, proteins involved in the biogenesis and maintenance of an integral outer membrane, regulators or proteins involved in QS (Huber et al., 2002).

Bacterial surface proteins

In two of the transposon mutants a gene encoding a large surface protein was inactivated. This gene was named *bapA* (biofilm associated protein A). Various members of this protein family were shown to be involved in the colonization of diverse substrates: Mus-20 (mutants unattached to seeds) in the adhesion of *P. putida* KT2440 to corn seeds (Espinosa-Urgel et al., 2000), Bap of *Staphylococcus aureus* in biofilm formation on mammary gland epithelium of ruminants (Cucarella et al., 2001), Esp (Enterococcal surface protein) of *Enterococcus faecalis* in human skin colonization (Toledo-Arana et al., 2001), *lapA* (large adhesion protein) of *P. fluorescens* in biofilm formation on abiotic surfaces (Hinsa et al., 2003), and BapA of *Salmonella enterica* in air-broth interface pellicle and biofilm formation (Latasa et al., 2005). These large proteins are usually secreted via a type I secretion system and are believed to remain loosely associated with the cell surface (Hinsa et al., 2003; Latasa et al., 2005). In a more recent study, a *bapA* mutant was found to be severely hampered in biofilm formation on an abiotic surface and showed defects in terms of attached biofilm mass as well as biofilm architecture (Inhülsen et al., 2012). BapA appears to be exported via a type I secretion system. The three genes encoding this export machinery are located downstream of *bapA* and are co-transcribed with *bapA* as a single transcription unit. A mutant with a mutated type I transporter exhibited the same biofilm defect as a *bapA* mutant and microscopic inspection of a strain expressing a BapA-mCherry fusion protein suggested that BapA is no longer exported (Inhülsen et al., 2012).

Although not identified in the mutant screen, an operon encoding three lectins (*bclACB*, BCAM0186 to BCAM0184) was found to influence biofilm structural development (Inhülsen et al., 2012). All three lectins share an almost identical PA-IIL-like C-terminal domain; BclB and BclC have additional N-

terminal domains. PA-IIL is a soluble lectin from *P. aeruginosa* with a strong affinity to fucose. A *P. aeruginosa* mutant not expressing PA-IIL is impaired in biofilm formation on glass slides when compared to the wild type strain (Winzer et al., 2000; Tielker et al., 2005). Two of the three lectins of *B. cenocepacia*, BclA (BCM0186) and BclC (BCAM0185) were subjects to recent studies (Lameignere et al., 2008, 2010; Sulák et al., 2010, 2011; Marchetti et al., 2012). These investigations revealed that BclA forms homodimers and displays a strict specificity for oligomannosetype oligosaccharides that are present on human glycoproteins (Lameignere et al., 2008; Marchetti et al., 2012). Moreover, it was shown that BclA binds to bacterial surfaces and to biofilms (Marchetti et al., 2012). BclC, which also contains the C-terminal PA-IIL domain, possesses in addition an N-terminal domain comprising a TNF-α-like fold with fucose binding properties. BclC forms hexamers and, due to its two different lectin domains, is a super lectin with dual carbohydrate specificity (Sulák et al., 2010, 2011). BclB is the least characterized of the three lectins encoded by *B. cenocepacia*. In a recent study it was shown that BclB is associated with the bacterial cell surface (Inhülsen et al., 2012). This study also revealed that the structure of the biofilm formed by a *bclACB* mutant is different from the one of the wild type. The mutant biofilm exhibited a very characteristic biofilm architecture structure with hollow microcolonies, which could not be observed with the wild type. This change in biofilm morphology could only be rescued when the complementation was done with the intact *bclACB* operon, suggesting that the three lectins are not redundant and that all three lectins are needed for biofilm structural development. The authors speculated that the surface-exposed BclACB lectins may mediate contact to neighboring cells within the biofilm or with the biofilm matrix.

Proteins involved in the biogenesis and maintenance of an integral outer membrane

Two genes essential for maintaining cell shape and outer membrane composition, *tolA* and *rodA,* were found to be required for biofilm formation. In *P. aeruginosa*, the homolog of *tolA* affects LPS structure and its expression was shown to be induced during biofilm formation (Whiteley et al., 2001). In addition, *tolA* is involved in the biogenesis and maintenance of an integral outer membrane in both *E. coli* and *P. putida* (Lazzaroni et al., 1999; Llamas et al., 2000). In agreement with this findings, *tolA* mutants of *B. cenocepacia* H111 were elongated and grew in chains, forming flat and unstructured biofilms (Huber et al., 2002). In contrast to *tolA* mutants, *rodA* mutants are coccoid and form hyper-structured biofilms with big microcolonies. Taken together, these data suggests that cell shape and cell surface composition are important factors for biofilm development.

Exopolysaccharide production

Exopolysaccharides (EPS) are considered to be an essential constituent of the biofilm matrix, even though the genes required for EPS biosynthesis were not identified in the mutant screen of Huber et al. (2002). The majority of Bcc strains produce large amounts of EPS and so far five different EPS molecules have been identified (Herasimenka et al., 2007). Among them, cepacian, a polysaccharide with a branched heptasaccharide repeating unit, is the most abundant EPS produced by Bcc species (Herasimenka et al., 2007). It has been shown that cepacian plays a role in biofilm maturation, specifically in the establishment of thick biofilms, but is not required for the initiation of biofilm formation (Cunha et al., 2004). The enzymes required for cepacian biosynthesis are encoded by two

separated gene clusters, *bce-I* and *bce-II* (Moreira et al., 2003; Ferreira et al., 2010). Interestingly, due to a frameshift mutation in the *bceB* gene (in the *bce-I* cluster), the ET12 lineage strain *B. cenocepacia* J2315, which is used as model strain in many laboratories, does not produce cepacian (Holden et al., 2009). Despite the fact that bacteria growing in biofilms have been shown to be more resistant to both antibiotics and phagocyte killing than their planktonic counterparts (Høiby et al., 2010), no clear correlation between cepacian production and the clinical outcome of the infection could be established when CF isolates of *B. cepacia*, *B. multivorans*, *B. cenocepacia*, and *B. stabilis* were analysed (Cunha et al., 2004). Zlosnik et al. (2008) reported an inverse correlation between the quantity of mucoid exopolysaccharide production by Bcc bacteria and rate of decline in CF lung function, which is opposite to what is known from *P. aeruginosa*. It has been suggested that non-mucoid isolates are associated with increased disease severity while the mucoid phenotype may be associated with bacterial persistence. In agreement with this hypothesis, it was shown that EPS from a clinical *B. cenocepacia* isolate inhibited the chemotaxis of neutrophils and scavenges reactive oxygen species, both essential components of innate neutrophil-mediated host defences (Bylund et al., 2006). In addition, it is possible that the loss of EPS production provides the bacteria with a competitive advantage in the lung and/or allows for increased virulence factor production (Zlosnik et al., 2008).

The role of QS in biofilm formation

A role for AHL-mediated QS in regulation of biofilm formation was first reported by Davies et al. (1998) for *P. aeruginosa*. Specifically, it was shown that a QS mutant formed flat and undifferentiated biofilms when compared to the wild type biofilm. Various QS-regulated functions, including the biosynthesis of rhamnolipids, the production of the biofilm matrix polysaccharide Pel, anaerobic denitrification, and *P. aeruginosa* quinolone signal (PQS)-dependent DNA release, have been identified as obvious links between biofilm structural development and cell–to-cell communication (for reviews, see Aguilar et al., 2009; de Kievit, 2009). Recent studies have shown that QS is necessary for the formation of the cap portion of the mushroom-shaped microcolonies, which are typical of *P. aeruginosa* biofilms (Yang et al., 2007, 2009; Patriquin et al., 2008). However, it is noteworthy that in studies using slightly changed experimental settings or hydrodynamic conditions, no significant differences between biofilms of the wild type and those formed by QS-negative mutants were observed (Stoodley et al., 1999; Heydorn et al., 2002; Schaber et al., 2007), suggesting that different experimental settings have a strong influence on expression of QS-regulated genes.

B. cenocepacia H111 was the second example of an bacterium that requires a functional AHL-dependent QS system for the formation of differentiated biofilms (Huber et al., 2001). While both wild type and mutants defective in the CepI/R QS system form characteristic microcolonies after initial attachment to an abiotic surface, striking differences can be observed in the later stages of biofilm development: the wild type biofilm rapidly matures whereas the QS-defective strains are arrested at the microcolony stage and thus form a thinner and less structured biofilm. The central importance of the CepI/R system in biofilm formation was also confirmed in a subsequent study, in which three mutants were identified that had insertions in *yciR*, *suhB* and *yciL*. As these mutants showed reduced levels of AHL, it was suggested that their biofilm defect is due to impaired expression of CepI/R (Huber

et al., 2001). In *B. cenocepacia* K56-2, the influence of the CepI/R and CciI/R QS systems on biofilm formation is rather complex: mutations in either *cepI* or *cepR* led to reduced amounts of biofilm; however, mutation of *cciI* or *cepI cciI* did not affect the ability to form a biofilm, while a *cciR* as well as a *cepR cciR* double mutant were deficient in biofilm formation (Tomlin et al., 2005). As with strain H111, higher-level regulators appear to fine-tune QS-dependent biofim development of strain K56-2. The sensor kinase hybrid AtsR, which is an important regulator of type VI secretion system and virulence factor expression, was shown to affect the timing of AHL production in this organism. This effect is thought to be the reason for the biofilm defect observed with an *atsR* mutant (Aubert et al., 2008).

The influence of QS on biofilm formation was confirmed for several *Burkholderia* species. A survey by Wopperer et al., (2006) has shown that the large majority of Bcc strains, belonging to nine species, formed biofilms in an AHL-dependent manner (Wopperer et al., 2006). In addition, mature biofilms of *B. cenocepacia* and *B. multivorans* were reduced upon treatment with QS-inhibitory compounds, reaffirming the importance of QS in the late stages of biofilm development (Brackman et al., 2009). Likewise, it has been shown that biofilm formation of *B. pseudomallei* is dependent on AHL signalling (Gamage et al., 2011).

In a recent study three AHL-regulated candidate gene clusters were tested for their role in biofilm formation of *B. cenocepacia* H111 (Inhülsen et al., 2012). The three loci were (i) the *bclACB* lectin operon, (ii) the large surface protein *bapA* and its transporter and (iii) a gene cluster encoding a type I pilus. This analysis revealed that BapA plays a major role in biofilm formation on abiotic surfaces while inactivation of the type I pilus showed little effect both in a static microtitre dish-based biofilm assay and in flow-through cells. Inactivation of the *bclACB* lectin genes gave rise to biofilms with an aberrant structure (see above).

The BDSF-dependent QS system of *B. cenocepacia* was also shown to have a strong effect on biofilm formation (Ryan et al., 2009; Deng et al., 2012). In fact, one of genes identified in the Huber et al. (2002) screen that are essential for biofilm formation, YciR, was recently identified as the main BDSF sensor and was renamed to RpfR (Deng et al., 2012; see above). Consistent with the results obtained by Huber et al. (2002), the reconstructed RpfR mutant was deficient in biofilm formation and phenocopied the defect of the BDSF synthase mutant (Deng et al., 2012). Most recent work has shown that the BDSF circuitry is also involved in the regulation of BapA expression (Schmid et al., 2012), providing a rationale for the biofilm defects observed with the $rpfF_{Bc}$ *or rpfR* mutants. Expression of *bapA* in *B. cenocepacia* H111 is therefore regulated by both the AHL- and the BDSF-dependent QS system. However, additional work is required to fully understand how these two signalling systems orchestrate the expression of this key factor for biofilm formation.

Interspecies and communication in mixed biofilms

Bcc strains and *P. aeruginosa* can form mixed biofilms in the lungs of CF patients. Given that both organisms control biofilm formation and expression of other virulence factors by QS, it appears likely

that the two species are capable of communicating with each other. Initial evidence for interspecies signalling was reported by McKenney et al. (1995), who showed that the production of siderophores, lipases and proteases by *B. cepacia* can be stimulated by the addition of concentrated *P. aeruginosa* PAO1 culture supernatants. Circumstantial evidence for an interaction of the two pathogens was also obtained when the longitudinal AHL profiles of *P. aeruginosa* isolated from chronically infected CF patients were analysed (Geisenberger et al., 2000). During the sampling period, two patients became co-infected with *B. cepacia*. While in one patient the observed AHL profile of *P. aeruginosa* isolates did not change, in the other case a dramatic reduction in the amount of AHL produced by *P. aeruginosa* was observed. Intriguingly, this reduction was only observed during the six-months period of co-infection. After clearance from *B. cepacia*, the *P. aeruginosa* AHL profile was resumed (Geisenberger et al., 2000). More direct evidence for AHL-mediated cross talk was obtained by the use of fluorescent biosensors, which were employed to visualize communication in mixed biofilms of *B. cenocepacia* H111 and clinical isolates of *P. aeruginosa* (Riedel et al., 2001). In the two biofilm model systems that were used, artificial flow chambers and alginate beads introduced into the lungs of mice, a *cepI* mutant of *B. cenocepacia* was able to respond to the AHLs released by *P. aeruginosa*. Interestingly, the opposite, namely *P. aeruginosa* responding to *B. cenocepacia*, was not observed (Riedel et al., 2001). In another study, however, it was demonstrated that *P. aeruginosa* and *B. cenocepacia* K56-2 were both able to utilize the heterologous signalling molecule (Lewenza et al., 2002), suggesting that communication between the two organisms is strain-dependent.

Evidence is accumulating that *cis*-2-unsaturated fatty acids of the DFS family may also be used for interspecies communication (Ryan et al., 2009). Many *Burkholderia* strains not only produce BDSF but some strains also synthesize DSF, the ancestral fatty acid signal isolated from Xcc, or other molecules of the DSF family (Deng et al., 2010). It is noteworthy in this context that DSF was shown to strongly bind to the BDSF RpfR receptor of *B. cenocepacia* and to be able to rescue the phenotypic defects of a BDSF mutant (Deng et al., 2012), suggesting that the two signals are interchangeable.

In a recent study Twomey et al. (2012) examined the role of DSF and BDSF in the regulation of virulence and persistence of *P. aeruginosa* in the cystic fibrosis airways. *P. aeruginosa* does not produce BDSF or DSF but the structurally related compound *cis*-2-decenoic acid, which was shown to induce the dispersal of biofilms (Davies and Marques, 2009). Twomey et al (2012) showed that the presence of DSF and BDSF in sputa of CF patients was correlated with the colonization of the patient with *B. cenocepacia* and/or *Stenotrophomonas maltophilia*, which is known to produce DSF (Fouhy et al., 2007). In the presence of DSF, persistence of *P. aeruginosa* was increased in a CFTR knockout mouse model. Furthermore, polymyxin tolerance was enhanced when *P. aeruginosa* was grown as a biofilm in the presence of DSF on human airway epithelial cells (Twomey et al., 2012). This study provided evidence that interspecies DSF-mediated bacterial interactions occur in the CF lung and it is tempting to speculate that this is also the case with BDSF, the main DSF family signal molecule produced by various *Burkholderia* species.

In addition to their roles in interspecies communication, DSF family signal molecules have also been implicated in interkingdom signalling. Many *cis*-2-unsaturated fatty acids, including DSF, BDSF and *cis,cis*-11-methyldodeca-2,5-dienoic acid, have been demonstrated to efficiently inhibit germ tube formation of the fungus *Candida albicans* (Boon et al., 2008; Deng et al., 2010).

QS as target for antibiofilm and antipathogenic drugs

QS as a target for the eradication of biofilms

Disruption of AHL-mediated QS systems has emerged as a promising strategy for the development of antimicrobial and antibiofilm drugs (Hentzer et al., 2002; Sokol et al., 2007). AHLs signalling molecules can be degraded enzymatically, a process known as quorum Quenching (Dong and Zhang, 2005; Dong et al., 2007). AHL inactivation can occur through AHL-acylases or AHL-lactonases. AHL-acylases degrade AHL by removing the fatty acid side chain from the homoserine lactone ring of AHLs (Dong et al., 2007) and have been reported to be produced by *Ralstonia* sp. (Lin et al., 2003; Chen et al., 2009), *Pseudomonas* spp. (Huang et al., 2006; Sio et al., 2006; Shepherd and Lindow, 2009; Wahjudi et al., 2011), *Streptomyces* sp. (Park et al., 2005), *Anabaena* sp. (Romero et al., 2008), and the Gram-positive *Rhodococcus erythropolys* (Uroz et al., 2005). Among these enzymes, there are some which prefer long-chain AHLs such as C_{10} to C_{14} (Huang et al., 2006; Sio et al., 2006). The second class of enzymes with AHL degrading activity includes AHL lactonases which have been identified in Gram-positive bacteria such as *Bacillus sp.* and *Arthrobacter* sp. (Dong et al., 2001, 2002; Park, 2003) and Gram-negative bacteria such as *Klebsiella* sp. and *Agrobacterium* sp. (Zhang et al., 2002; Park, 2003). Heterologous expression of the AHL lactonase *aiiA* from *Bacillus* spp. in *Pseudomonas* or *Burkholderia* resulted in a marked decrease in the amount of AHL signals produced (Dong et al., 2000; Reimmann et al., 2002). It was recently shown that *Bacillus* sp. isolates which show quorum quenching activity were able to impair biofilm growth in *B. pseudomallei* (Ramli et al., 2012). Heterologous expression of the AiiA lactonase in different *B. cepacia* complex (Bcc) species was used to demonstrate that some QS-regulated functions, including protease production, biofilm formation and nematode pathogenicity, are highly conserved among Bcc strains (Wopperer et al., 2006). Another strategy to interrupt QS is by means of signal antagonists, the so-called QS inhibitors (QSIs) (Rasmussen and Givskov, 2006). To date, many QSIs have been identified, some of which have been isolated from natural products, such as halogenated furanones isolated from the marine alga *Delisea pulchra* (Givskov et al., 1996) and 4-nitro-pyridine-N-oxide from garlic (Rasmussen et al., 2005a). The furanone compound C-30 was shown to inhibit biofilm formation and promote a faster clearance of *P. aeruginosa* lung infection in mice (Hentzer et al., 2002; Wu et al., 2004). Furanones are thought to stimulate degradation of LuxR receptor proteins by cellular proteases (Manefield et al., 2002) and to compete with AHL for binding to LuxR (Müh et al., 2006). Other compounds such as a sulfur-rich molecule from garlic ajoene (Jakobsen et al., 2012b), iberin from horseradish (Jakobsen et al., 2012a), patulin and penicillic acid from fungi (Rasmussen et al., 2005b) have been shown to have QSI activity and to exhibit antimicrobial activity against a number of Gram-positive and Gram-negative bacteria (Naganawa et al., 1996).

A rational drug design approach in combination with a high-throughput screening using AHL biosensors was employed by Riedel et al. (2006) to develop QS blockers of the CepI/R QS system of

B. cenocepacia. This approach lead to the identification of a compound that is entirely unrelated to AHLs and furanones but interferes with the expression of QS-regulated functions such as proteases, swarming motility and biofilm formation of *B. cenocepacia*, presumably via a competitive inhibition mechanism. Recent work confirmed the efficacy of this compound on biofilm formation of *B. cenocepacia* and *B. multivorans* (Brackman et al., 2009). Although the clinical performance of QSI compounds remains to be seen, such drugs show great promise, either alone or in combination with conventional antimicrobial agents, for treatment of *Burkholderia* sp. infections.

Future directions

Members of the genus *Burkholderia* have emerged as multilingual talents that were demonstrated to master several bacterial languages. AHL-mediated communication is probably the most widespread and certainly the best investigated cell-to-cell signaling system operating in members of this genus. Work of the past few years has identified many AHL-controlled functions in diverse *Burkholderia* species. These investigations revealed a core set of highly conserved AHL-regulated functions that include biofilm formation, proteolytic activity, motility, antifungal activity and pathogenicity. The more recently discovered BDSF signalling system was also shown to be involved in the regulation of biofilm formation and virulence and evidence has been presented that the two QS regulons partly overlap (Schmid et al 2012). A main direction for future work will be a detailed analysis of connections between the two regulatory cascades. We have recently presented a working model of the QS circuitry of *B. cenocepacia* (Schmid et al., 2012). In this model we assume a yet unidentified c-di-GMP receptor protein X, the activity of which is directly controlled by the phosphodiesterase activity of the BDSF sensor RpfR. Protein X is thought to be required for transcription of target genes either directly or via additional regulators. Although the two QS systems could operate independently of each other, we favor the idea that the two regulatory cascades converge and control the expression or the activity status of an unknown common regulator Y, which in turn regulates expression of target genes (Figure 3). Additional work will be required to distinguish between the two possibilities and to identify the responsible c-di-GMP effector.

The intracellular secondary messenger c-di-GMP is considered to be a key signal for the bacterial lifestyle, with high levels being typical for sessile cells during chronic infections and low levels for planktonic cells and acute infections (Jonas et al., 2009). Besides the initial investigations on the effects of the BDSF receptor RpfR on intracellular c-di-GMP levels very little work was done to investigate the role of this secondary messenger in virulence and biofilm formation in *Burkholderia* sp. In *B. pseudomallei* a cyclic di-GMP phosphodiesterase, CdpA, was identified and shown to be involved in autoaggregation, motility, biofilm formation, cell invasion, and cytotoxicity (Lee et al., 2010). For *B. cenocepacia* it has been demonstrated that artificially elevated intracellular levels of c-di-GMP promoted wrinkly colony, pellicle and biofilm formation (Fazli et al., 2011). In this study a transcriptional regulator, BCAM 1349, was identified that can bind c-di-GMP and is involved in the production of a number of biofilm matrix components, including cellulose and fimbriae. We believe that these anecdotal reports are just the tip of the iceberg of a new emerging role of c-di-GMP in controlling pathogenicity and the sessile lifestyle of members of the genus *Burkholderia*.

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Figures & Figure legends

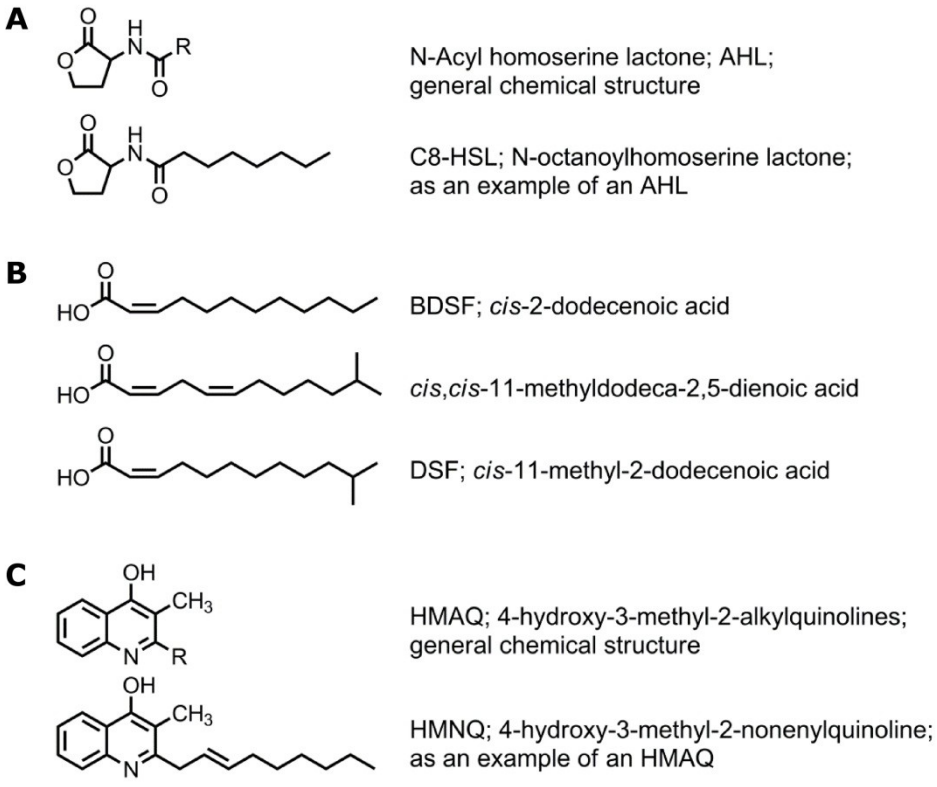

Figure 1: The different QS signalling molecules used by *Burkholderia* species. A: Signalling molecules in AHL-based QS. B: Signalling molecules in fatty acid based QS. C: Signalling molecules in quinoline-based QS.

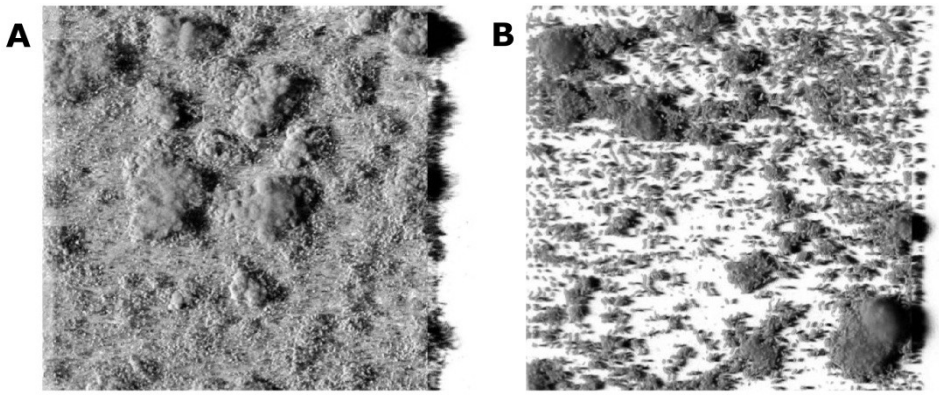

Figure 2: Simulated 3-D view of a 48 h old biofilm of *B. cenocepacia* H111. A: wild type. B: isogenic double mutant defective in both *cepI* (AHL synthase) and $rpfF_{Bc}$ (BDSF synthase).

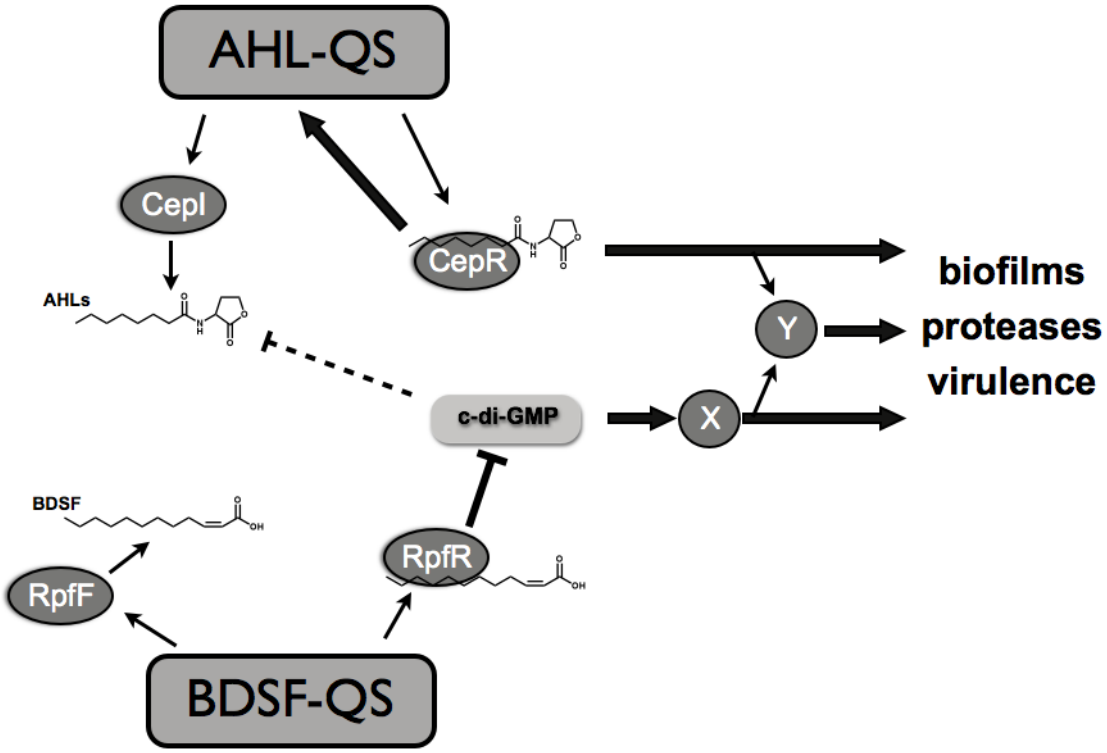

Figure 3. Model for the possible interactions of the AHL- and BDSF-dependent QS systems of *B. cenocepacia* H111 QS. The CepI/R system consists of the AHL synthase CepI directing the synthesis of C8-HSL, and of the transcriptional regulator CepR. The RpfF/RpfR system consists of RpfF which directs the synthesis of BDSF, and of its cognate receptor RpfR, which upon binding of BDSF degrades c-di-GMP. The two QS systems operate in parallel and control specific as well as overlapping sets of genes. We propose the existence of a c-di-GMP receptor protein X that stimulates transcription of target genes independent of CepR. However, the two QS cascades could also converge and control the expression or the activity status of an unknown common regulator Y, which in turn regulates expression of target genes. C-di-GMP has a negative regulatory effect on AHL levels via an unknown mechanism (depicted by the dashed line).

Table 1: Experimentally determined phenotypes of QS mutants in *Burkholderia* species.

Species[a]	Mutated QS system	Biofilm[c]	Protease	Chitinase	Lipase	Siderophore	Swarming motility	Swimming motility	Antifungal activity	Pathogenicity: *C. elegans*	Pathogenicity: Galleria	Pathogenicity: mouse/rat/hamster	Pathogenicity: zebrafish	Pathogenicity: alfalfa	other QS regulated phenotypes	Reference:
B. ambifaria	*cepI/R*	↓[d]	↓						↓	↓	↓					(Wopperer et al., 2006; Uehlinger et al., 2009)
	HMAQ		↑			↑			↑							(Vial et al., 2008)
B. anthina	*cepI/R*	↓	↓ (1), – (1)				↓			↓						(Wopperer et al., 2006)
B. cenocepacia with cci	*cepI/R*	↓	↓		↓	↑	↓	↓		↓	↔	↓	↓	↔		(Lewenza et al., 1999; Sokol, 2003; Malott et al., 2005; Tomlin et al., 2005; O'Grady et al., 2009; Uehlinger et al., 2009; Vergunst et al., 2010)
	cciI/R	↓ (cciR), ↔ (cciI)	↑ (cciI), ↓ (cciR)			↔	↓ (cciI), ↔ (cciR)	↑		↔	↔	↓		↔		(Malott et al., 2005; Tomlin et al., 2005; O'Grady et al., 2009; Uehlinger et al., 2009)
	rpfF/R	↓					↓				↓	↓	↓		germtube formation in *Candida albicans*:↓ ; adherence to porcine mucin: ↓, cytotoxicity: ↓	(Boon et al., 2008; Deng et al., 2009; Ryan et al., 2009; McCarthy et al., 2010)
B. cenocepacia without cci	*cepI/R*	↓	↓	↓	↔	↑	↓	↔	↓	↓	↓ cepI, ↔ cepR	↓, ↔[f]				(Huber et al., 2001; Köthe et al., 2003; Sousa et al., 2007; Schmidt et al., 2009; Uehlinger et al., 2009)
	rpfF/R	↓	↓				↓			↓						(Deng et al., 2012)
B. cepacia	*cepI/R*	↓	↓	←	↔	↔			↓	↓					onion pathogenicity: ↓	(Aguilar et al., 2003; Wopperer et al., 2006; Schmidt et al., 2009)
B. dolosa	*cepI/R*	↓	–							↓ (1), – (1)						(Wopperer et al., 2006)
B. lata	*cepI/R*					↔			↓							(Schmidt et al., 2009)
B. multivorans	*cepI/R*	↓	–							– (6), ↓(1)						(Wopperer et al., 2006)
B. pyrrocinia	*cepI/R*	↓(1), ↑ (1)	↓						↓	↓						(Wopperer et al., 2006; Schmidt et al., 2009)
B. stabilis	*cepI/R*	↓	↓				↓		↓	↓						(Wopperer et al., 2006; Schmidt et al., 2009)
B. vietnamiensis	*cepI/R*	↓(3), ↑(2), ↔ (1)	–		–	↔	–		↓	↓ (3), – (2)	↓			–		(Conway and Greenberg, 2002; Wopperer et al., 2006; Schmidt et

	bviI/R	↔	–	–	↔	–			–		al., 2009; Uehlinger et al., 2009) (Malott and Sokol, 2007)
B. pseudomallei	*pmlI/R or bpsI/R*	↓	↑, ↔ [e]	↔	↑			↓	↓	Sensitivity to oxidative stress: ↑	(Ulrich et al., 2004a; Valade et al., 2004; Song et al., 2005; Lumjiaktase et al., 2006; Gamage et al., 2011)
B. thailandesis	*btaI/R*		↔	↓, ↑	↔	↑			↔	bactobilin synthesis ↓, selfaggregation ↓	(Ulrich et al., 2004c; Chandler et al., 2009; Duerkop et al., 2009; Seyedsayamdost et al., 2010)
B. mallei	*bmalI/R*		↔	↔					↓		(Ulrich et al., 2004b)
B. glumae	*TofI/R*			↓		↓	↓			catalase: ↓, toxoflavin synthesis: ↓, heat shock resistance ↓, rice pathogenicity: ↓	(Kim et al., 2004, 2007, 2012; Devescovi et al., 2007; Chun et al., 2009)
B. plantarii	*PlaI/R*									rice seedling blight ↓	(Solis et al., 2006)
B. kururiensis	*BraI/R*	↑	↔	↔	↔	↔	↔			EPS production: ↓	(Suárez-Moreno et al., 2008, 2010)
B. xenovorans	*BraI/R*	↔	↔	↔	↔	↔	↔			EPS production: ↓	(Suárez-Moreno et al., 2008, 2010)
B. unamae	*BraI/R*	↑								EPS production: ↓, phenol degradation: ↓	(Suárez-Moreno et al., 2008, 2010)
B. caryophylii	AHL[b]							↓			(Schmidt et al., 2009)
B. phenaziniu m	AHL[b]							↓			(Schmidt et al., 2009)
B. bryophila	AHL[b]							↓			(Schmidt et al., 2009)
B. megapolitan a	AHL[b]							↓			(Schmidt et al., 2009)

[a] *Burkholderia* species, in which a QS regulated phenotype has been observed.
[b] The phenotypic testing has been performed with an AHL degrading approach.
[c] Number in parentheses indicates the number of strains in which the according regulation has been observed if a phenotype is strain-dependent.
[d] ↓ down-regulated in QS mutant, ↑ up-regulated in QS mutant, ↔ not regulated in QS mutant (experimentally verified), – phenotype not observed in wild type strain.
[e] Opposing observations in different studies.
[f] Reciprocally by the different QS systems: btaI/R1 and btaI/R3 ↑, btaI/R2.

8.3 Nucleotide sequence of *iclR*

The insertion of the transposons took place between the underlined bases.

>ENA|CCE51534|CCE51534.1 Burkholderia cenocepacia H111 transcriptional regulator, IclR family :

GTGTCGACAACTGTAACCCCTCCCGCGTCGCGGGACCGTGAATCGTCACCCGACGAGATCACCGCCCTCGCCCGCGGCCTCGCCGTGCTGC
GCCGTATCGCGGCTGCCGACGCGCCCGTCAGCAACCGCGAACTGACCGAATTGACCGGCATCCCGAAGCCGACCGTCTCGCGCATCACCGC
GACGCTCGTCAGCGCCGGCTTCCTGTTCCAGTTGCCCGACAGCGAGCGCTTCGTGCTCACCGCGTCGGTGCTCGAACTGAGCCACGGCTTC
CTGCGCAACTTCGACATCCGCGCGCGTTCGCGGCCGTTCATGATCGAGCTGGCCGAACGCACGTCGCTGTCCGTGCACCTCGCGGTGCGCG
ACCGCCTCGACATGGTCGCGATCGACGTGATCCGCCCGCGCTCGGCCGTGCTCGTCACGCGCCTCGAGATCGGCTCGCGGATGGACATCGC
GCGTACGGCCGTCGGCCGCGCGTATCTCGCCGCGCTGGAGGACGACGAGCGTCGCCTGCTGCTCGAATCGCTGCGCACCACCGCCGGCGAC
GACTGGCCGCACGTGTCCGCTCGCCTCACGCCCGCGCTCGACGAAGCGATGCGCGACGGCCATGCGATCGCGATCGGCGAATGGCGCGAGG
GCCTCAATGCGGTCGCGGCCGGTTTCGTCGGCCCGTCGGGTCAACGCTATTCGGTGAATTGCGGCGGCGCGTCGCACCAGTGCCCGCCCGA
ATGGCTGCAGGAACACGTGGTGCCCGCGCTGCAGGAATGCATCGCGAAAATCACCCGCGAAATCGGCGGCGCGCCGGCCCGGCGCATCGGC
GTGTAA

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Deng Y*, Schmid N*, Wang C, Wang J, Pessi G, Wu D, Lee J, Aguilar C, Ahrens CH, Chang C, Song H, Eberl L, Zhang L-H. *Cis*-2-dodecenoic acid receptor RpfR links quorum-sensing signal perception with regulation of virulence through cyclic dimeric guanosine monophosphate turnover. Proc Natl Acad Sci U S A. 2012;109(38):15479–84.

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*: equally contributed

Conference contributions

Oral presentations

April 2013 Ann Arbor MI, USA	International Burkholderia cepacia Working Group, 17th Annual Meeting	New insights into the BDSF- and the AHL-based quorum sensing systems of *Burkholderia cenocepacia* H111
April 2011, Prague, Czech Republic	International Burkholderia cepacia Working Group, 15th Annual Meeting	Factors linking quorum sensing and biofilm formation in *Burkholderia cenocepacia* H111

Posters

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June 2013, Interlaken, Switzerland	71th Annual assembly of the Swiss Society for Microbiology (SSM)	New insights into the BDSF- and the AHL-based quorum sensing systems of *Burkholderia cenocepacia* H111

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